FEB 3 1921

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME XXXIII, NO. 2 FEBRUARY 1, 1921

PUBLISHED MONTHLY

AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917.
Authorized June 29, 1918.

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

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WAVERLY PRESS
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BALTIMORE, U. S. A.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

I. Transmission Experiments with Nasopharyngeal Washings.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 5 to 7.

(Received for publication, July 15, 1920.)

INTRODUCTION.

In planning the present experiments we had in mind the possible presence in the nasopharynx of persons suffering from acute epidemic influenza of some agent the effects of which might be noted in animals. In considering the criteria of activity of this agent we thought, first, of the well known phenomenon in man of leucocytic depression, involving especially the mononuclear cells, during the acute influenzal attack, and next, of changes of a more or less pronounced but possibly transient character, arising in the lungs, which might conceivably predispose to the severe pneumonias that often accompany as a secondary or concurrent infection the influenzal attack.

Furthermore, this study was made during the course of over $1\frac{1}{2}$ years in three successive periods. The first period coincided with the epidemic wave of 1918–19. During this period cases of acute uncomplicated influenza and individuals who had never been affected were studied. The second period embraced the late autumn of 1919, during which influenza did not prevail in New York in epidemic form. During this interepidemic period normal individuals were studied as controls. The third period, the winter of 1920, saw a return of the epidemic. At this time additional cases of the disease were available for investigation. By proceeding in this manner we hoped to check the results for each period against the others. As the sequel will show, we believe that we succeeded in this undertaking, with the consequence that we are enabled to present our findings with perhaps a degree of confidence not otherwise appropriate.

Materials.

The outstanding difficulty in the choice of materials to be employed arose from the necessity of selecting cases of undoubted acute influenza, on the one hand, and of perfectly healthy individuals, who had never suffered from the disease, on the other. In the end the second requirement was more easily fulfilled, as the circumstances of the undertaking admitted of leisurely and painstaking choice of subjects. With uncomplicated influenza, however, the individuals had to be chosen at once, since the epidemic wave of the disease is notably brief, being prolonged chiefly by secondary respiratory infections.

The criteria which were used as guides in the selection of cases of pure influenza were abrupt onset with chilliness, fever, prostration, headache, and muscular pains, especially in the back and limbs. Among the early symptoms were flush and suffusion of the face, injection of the conjunctivæ, soreness of the throat, and harsh, unproductive cough. In the early stages no physical signs were detected in the chest, gastrointestinal symptoms were inconspicuous, and disturbances referable to other internal organs were not complained of or detected by physical examination.

These symptoms, although striking, were rarely such as could be measured accurately. However, there was one sign that had a quantitative value; namely, the leucocytic picture. Uncomplicated influenza shows a pronounced leucopenia affecting the absolute number of mononuclear cells, chiefly of the lymphocytic variety.[1] This is persistent and even resists at times secondary infectious processes, *e.g.* pneumonia, in which leucocytosis is the rule. As will appear below, great reliance was placed on this quantitative sign in the present experimental studies.

The symptoms and effects endured for from 1 to 3 days, when convalescence, initiated by a lytic fall of temperature, set in, and recovery promptly followed.

[1] The term mononuclears as employed by us includes the leucocytes of the lymphocytic and large mononuclear varieties which have a single homogeneous nucleus. Any indentation of the outline of the nucleus placed the cell in the transitional class, to be counted with the polymorphonuclear cells. Of the varieties of mononuclear cells, the small cells, or lymphocytes, were especially involved in the leucopenia.

Saline washings from the nose and throat were employed. These materials were secured from eight cases of influenza within the first 36 hours of the disease, and from twelve cases at later stages, including the convalescence or the period of postinfluenzal pneumonia. In addition, fourteen individuals who had not been affected were tested during the epidemic or interepidemic period.

Choice of Animal.

In earlier experiments, having in mind a filterable microorganism or virus, we employed *rhesus* monkeys. But this species of animal was found to be unsatisfactory. Monkeys are at best scarce in this country and frequently suffer from pulmonary lesions of a tubercular or other type; the experiments required animals more readily available and free from respiratory affections of any nature. The rabbit was therefore chosen.

EXPERIMENTAL.

Full grown rabbits were used for inoculation, and no rabbit suffering from snuffles or any detectable disease was employed. All animals were subjected to preliminary blood-counting, weighing, and temperature-taking, and any showing variations beyond the average were rejected.[2] These observations were made on 3 to 7 successive days previous to inoculation. The blood counts were carefully controlled; separate apparatus were used for each animal, which was examined throughout the course of observation by the same technician. Particular stress was laid on the total number of leucocytes and the relative and absolute numbers of polymorphonuclear and mononuclear cells.[1] With regard to temperature, it should be emphasized that in these animals the temperature in itself is no indication at times of the extent or absence of pathological involvement but should be interpreted only in conjunction with other findings.

[2] For assistance in this and other work we acknowledge our indebtedness to Captain Frank Hornaday, Medical Corps, U. S. Army, and to the Army Laboratory Technicians Miss Mary Jardine, Miss Clara M. McKee, and Miss Anne Webb.

Mode of Inoculation.—The inoculations were made directly into the lungs by means of the intratracheal catheter—a method slightly modified from that employed by Lamar and Meltzer[3]—or by tracheotomy.[4]

The first method, having the advantage of rapidity of operation, consisted in the insertion intratracheally by way of the mouth of a French silk catheter, size 9. An electric otoscope set in the mouth of the animal served as a gag as well as a guide for directing the catheter. The catheter was curved at 90° ½ inch from the tip. In inserting one should avoid slipping over the larynx into the esophagus, thereby contaminating the catheter.

Although tracheotomy requires more time, it is the preferable procedure because contamination by mouth bacteria is avoided. An incision is made directly over the trachea, and a needle, size 19, bent at a right angle, is inserted therein.

Materials Inoculated.—The materials employed for inoculation consisted of (*a*) unfiltered nasopharyngeal washings,[5] (*b*) filtered washings, (*c*) lung tissue suspensions,[6] filtered and unfiltered, from previously inoculated rabbits, (*d*) similar lung tissue preserved in sterile 50 per cent glycerol, (*e*) bacteria and culture materials, and (*f*) control materials. The usual dose for a 2.5 to 3 kilo rabbit was 3 cc. of these materials.

Unfiltered nasal washings were employed in the expectation that they could be purified, or rather deprived of their ordinary bacteria by successive animal passages. It was believed that if this could be accomplished there would be a better chance of preserving and possibly of causing the multiplication of some other variety of microorgan-

[3] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

[4] With either method light ether anesthesia was given.

[5] Nasopharyngeal washings were obtained as follows: The patient's mouth was rinsed with warm saline solution. Then each nasal cavity was washed with 25 to 35 cc. of saline solution, the washings being returned by way of the mouth and collected in a sterile container. The entire fluid was shaken with glass beads in a mechanical shaker for 15 minutes at high speed, or until a homogeneous mixture resulted.

[6] Lung tissue was prepared for inoculation as follows: The selected portion of the lungs was chopped and then ground with sterile, fine, white sand, and a proportional amount of saline solution was added in the ratio of 20 cc. of saline solution to the two entire lungs. The suspension was then centrifuged at low speed and the clearer supernatant fluid employed for inoculation.

ism, more resistant and virulent perhaps, which would give to the washings from cases of uncomplicated influenza a quality lacking in others. If was, of course, realized that not in every instance could this favorable outcome be looked for. Now and then it was to be expected that a virulent pneumococcus or streptococcus would set up a pneumonia to which the animal would succumb. But if the ordinary bacteria could be suppressed by animal passages in a few instances and something survive which produced definite changes in the tissues of the rabbits—the blood and lungs, for example—the washings from cases of influenza might thus be distinguished in their effects from the washings of another origin. In this way the operation of a pathogenic agent is to be deduced, although it might not be possible to determine certainly that this agent is the inciting microbic agent of influenza. However, if a certain correspondence in tissue and other effects can be shown to exist between the individual suffering from influenza and the rabbit inoculated with materials originally derived from influenza cases and free from all ordinary bacteria, an idea as to the probable nature of the pathogenic agent is gained which encourages further investigation along the indicated lines.

Inoculation of Unfiltered Washings.

There were inoculated into the lungs of rabbits the unfiltered nasopharyngeal secretions derived from five cases of uncomplicated influenza during the first epidemic, and three during the second, in the first 36 hours of the disease, and from eleven cases during the first epidemic and one during the second in the later stages of the affection. The following effects were induced by the materials from seven of the eight early cases, but not by any from the twelve cases examined after 36 hours.

Clinical Effects.—From 24 to 48 hours after inoculation fever developed, associated with the ordinary signs of indisposition in a rabbit, such as listlessness and ruffled hair, and conjunctivitis. The striking feature, however, was the definite and often marked leucopenia resulting from depression of the mononuclear cells, as shown in Text-fig. 1, *a*, *b*, and *c*. If the condition was allowed to run its natural course, these symptoms endured for about 3 days, the animal then returning to

normal. If the rabbit was killed—for in the absence of infection by ordinary bacteria none died—an unusual pathological picture was revealed.

Pathological Effects.—The respiratory organs were affected to the exclusion of all others. No pleuritis or exudate in the pleural cavity was evident. The lungs were voluminous as a result of edema and emphysema and had a mottled hemorrhagic appearance. The hemorrhages on the surface, beneath the pleura, were diffuse or discrete, occupying areas a few millimeters in extent or covering a large part of a lobe. In addition, minute petechiæ were seen scattered over the entire surface. On section of the lungs the cut surface revealed a hemorrhagic edema; it dripped a blood-stained, frothy fluid. The hemorrhages again were either diffuse and large, or discrete and small, in the latter instance being numerous.

On microscopic section carried through various parts of the lungs the lesions were found to consist (*a*) of hemorrhagic foci, and (*b*) of edema and emphysema. The hemorrhages varied in size in accordance with the observed macroscopic appearance, some being microscopic in nature. The edema was more extensive than the hemorrhages and involved alveoli and interalveolar strands of tissue. The alveoli contained coagulated serum or red corpuscles, mononuclear cells, and also at times polymorphonuclear cells of eosinophilic type and desquamated epithelial cells. The interalveolar strands were infiltrated with mononuclear cells and large cells the foreign nature of which was not always clear. Fibrin was sometimes present in small amounts. The bronchi, also, were at times filled with erythrocytes, exfoliated and degenerated epithelia, and leucocytes. The capillaries were distended with blood.

No ordinary bacteria were seen in impression films of the lung tissue or in sections stained by Gram's or MacCallum's[7] method, or in aerobic or anaerobic cultures of the tissue.

The two following protocols are presented in order to show the clinical and pathological effects, regarded as typical, which arise independently of the presence of ordinary bacteria of any demonstrable kind.

[7] MacCallum, W. G., The pathology of the pneumonia in the United States Army camps during the winter of 1917–18, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919, 47.

PROTOCOL 1.

Patient 8.—M. T., adult female. Feb. 9, 1919. Onset sudden with chills and fever. Feb. 10. Photophobia; prostration; muscular pains in the back and extremities; mild unproductive cough. Temperature 37.8°C.; pulse 80. Leucocytes 8,725, of which 1,832 were mononuclears. Physical examination showed lungs to be clear; pharynx red and congested; conjunctivæ injected. No other organs affected. Cultivation of sputum and nasopharyngeal washings yielded Pneumococcus Type IV; no Pfeiffer bacilli. The fever persisted for 48 hours and was followed by an uneventful recovery.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 20 hours after the onset of the symptoms were inoculated intratracheally into Rabbit A.[8]

First Passage. Rabbit A.—Before injection the normal blood counts showed the following results: Feb. 8, 1919. Leucocytes 13,050, of which there were 6,525 each of polymorphonuclears and mononuclears. Feb. 10. Prior to inoculation, leucocytes 14,425, of which 7,357 were polymorphonuclears and 7,068 mononuclears. Temperature 39.5°C. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 8. Feb. 11. Conjunctivitis. Leucocytes 12,550, of which 7,781 were polymorphonuclears and 4,769 mononuclears. Temperature 39.6°C. Feb. 12. Conjunctivitis persists. Leucocytes 11,450, of which 7,786 were polymorphonuclears and 3,664 mononuclears. Temperature 39.8°C.

The development within 24 hours of the conjunctivitis, the rise in temperature with depression of the leucocytes and mononuclears, which endured for 48 hours, were indications for killing the animal. Hence on Feb. 12 the animal was killed.[9]

Autopsy.—The lung condition was such as has been described as a typical effect of the inoculation.

Aerobic Cultures.—Cultures on the usual media remained sterile.[10]

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[8] Usually more than one rabbit was inoculated at the same time, but in this instance only the course of the rabbit used for further transmission experiments is described.

[9] In all experiments the animal was killed by a sharp blow, which dislocated the upper cervical vertebræ. In this way the complicating effects of ether anesthesia on the respiratory tract were avoided. The blow should be properly directed; otherwise the chest may be struck, thus causing contusion of the lung, or the skull may be broken with consequent profuse hemorrhages and aspiration of the blood, in which event the pathological picture is obscured.

[10] As a routine practice aerobic blood cultures were made before the animal was killed. Also pieces of lung tissue were planted in 1 per cent dextrose broth, and in this medium plus rabbit blood. Anaerobic cultures of lung tissue will be described in another communication.

Second Passage. Rabbit B.—Average normal leucocyte count before inoculation 10,775, of which 5,604 were mononuclears. Feb. 12, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit A. Feb. 13. Leucocytes 7,600, of which 3,268 were mononuclears. Feb. 14. Killed.

Autopsy.—The lung appearance was typical.

Aerobic Cultures.—Remained sterile.

Third Passage. Rabbit C.—Average normal leucocyte count before inoculation 15,060, of which 7,461 were mononuclears. Feb. 14, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit B. Feb. 15. Leucocytes 5,675, of which 2,043 were mononuclears. Feb. 16. Leucocytes 8,760, of which 3,942 were mononuclears. Killed.

Autopsy.—Typical lung lesions, but to a milder degree.

Aerobic Cultures.—No growth of ordinary bacteria obtained.

A suspension of the lung tissue from this rabbit failed, however, to produce a similar effect in the succeeding rabbit. At this point the series of transmissions was terminated.

PROTOCOL 2.

Patient 16.—N. L., adult female. Mar. 28, 1919. Onset sudden with chills and fever. Mar. 29. Temperature 39°C.; muscular pains in back; prostration and weakness; epistaxis; unproductive cough; coryza and photophobia. Flushed face; injected conjunctivæ; congested pharynx; lungs, a few râles anteriorly and posteriorly on left side only. No other organs affected. Leucocytes 7,300; mononuclears 2,117. Cultivation of sputum and nasopharyngeal washings yielded Pneumococcus Type IV. Sputum injected into mouse yielded no Pfeiffer bacilli, only the pneumococci. On the 2nd day of illness the temperature declined, and the general condition improved. On the 3rd day a relapse occurred, but recovery began on the 4th day.

Animal Inoculation.—The unfiltered nasopharyngeal washings were obtained 36 hours after the onset and were inoculated into the lungs of Rabbit A.

First Passage. Rabbit A.—Mar. 29, 1919. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 16. Mar. 30. Conjunctivitis. Leucocytes decreased from the norm of 8,200 to 6,700, and mononuclears from 4,100 to 2,680. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—Free from growth.

Second to Sixth Passages. Rabbits B, C, D, E, and F.—Each rabbit was injected similarly with a suspension of the lung tissue of the immediately preceding rabbit of the series. All showed uniform clinical effects. After 24 to 48 hours there developed conjunctivitis, leucopenia, mononuclear depression, and varying temperature reactions. None died, but all were killed 48 hours after the injection, except Rabbit C which was killed after 24 hours.

Autopsy.—There were present varying degrees of the typical lung lesions. The lung lesions of the last (sixth) rabbit passage are shown in Figs. 1, 3, and 4.

Aerobic Cultures.—In each instance free from growth.

Further transmissions were not made and hence this series terminated in the sixth rabbit passage.

The two series of experiments recorded in Protocols 1 and 2 indicate that definite and consistent clinical and pathological effects were induced in two series of rabbits with materials derived from the nasopharynx of recent acute cases of influenza which were independent of the presence of ordinary aerobic bacteria.

The next series of three protocols is given in order to bring out the fact that the clinical and pathological effects regarded as typical appear even when ordinary bacteria are cultivable, and also that these bacteria are suppressible through successive inoculation while the typical effects continue to occur.

PROTOCOL 3.

Patient 6.—K., adult female. Jan. 27, 1919. Onset sudden with chills and fever. Jan. 28. Pains in the muscles of back and legs; prostrated and weak; severe frontal headache; mild unproductive cough. Temperature 38°C. Physical examination showed no signs in chest; conjunctivæ injected. Cultivation of sputum and nasopharyngeal washings yielded Pfeiffer bacilli and Pneumococcus Type IV. Symptoms persisted for 3 days, followed by an uneventful recovery.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 24 hours after the onset of the symptoms were inoculated into the trachea of Rabbit A.

First Passage. Rabbit A (Text-Fig. 1, a).—Jan. 28, 1919. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 6. Blood count of rabbit prior to inoculation showed 15,025 leucocytes, of which 8,414 were mononuclears. Jan. 29. Purulent conjunctivitis. Leucocytes 6,600, of which 2,244 were mononuclears. Jan. 30. Leucocytes 8,025, of which 2,006 were mononuclears. Killed.

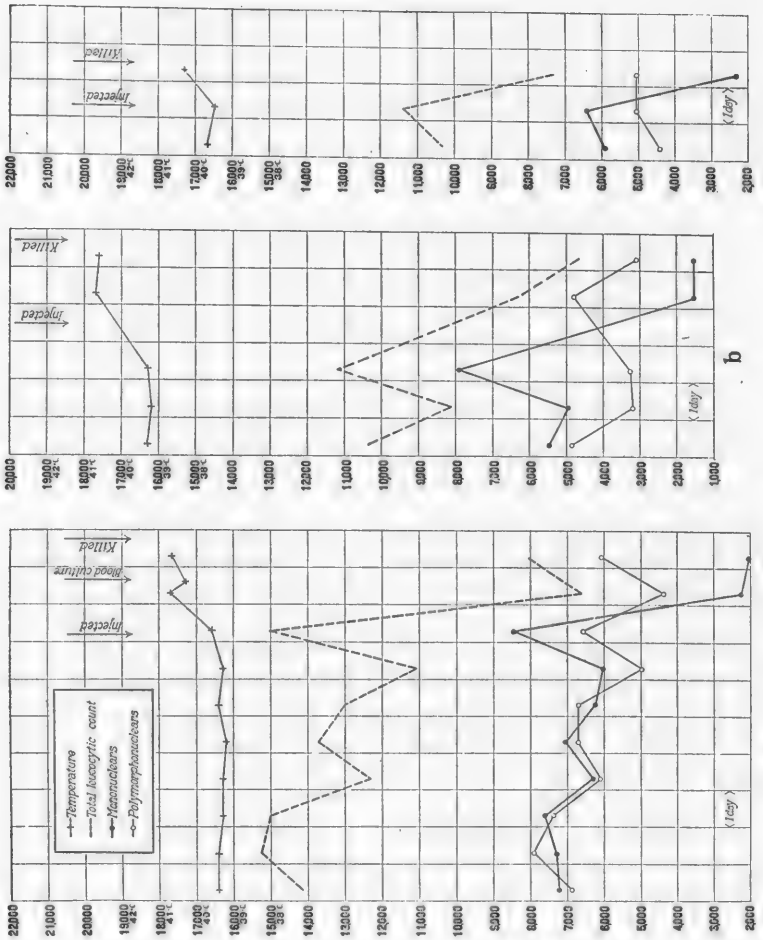
Autopsy.—Left lung showed gray hepatization with fibrinous pleuritis (lobar pneumonia); right lung, aside from a small area of atelectasis in upper lobe, showed edema and emphysema with large numbers of petechial hemorrhages.

Aerobic Cultures.—Left lung yielded a growth of Pneumococcus Type IV, avirulent for mice;[11] right lung yielded no growth of ordinary bacteria.

The right lung was employed for inoculation into the next rabbit.

Second Passage. Rabbit B (Text-Fig. 1, b).—Jan. 30, 1919. Injected intratracheally with 3 cc. of the suspension of tissue from right lung of Rabbit A.

[11] The significance of the ordinary bacteria encountered in these experiments will be dealt with in another communication.



TEXT-FIG. 1, *a*, *b*, and *c*. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. (*a*) First rabbit passage of the nasopharyngeal washings from a case in the early stage of uncomplicated influenza (Patient 6). (*b*) Second rabbit passage from the same case. (*c*) Seventh rabbit passage from Patient 11.

Feb. 1. Killed after having shown a depression of 6,765 in the total count and 6,269 in the mononuclear count on the 2nd day after injection.

Autopsy.—Both lungs were edematous and emphysematous and showed multiple punctate hemorrhages.

Aerobic Cultures.—No growth obtained from heart's blood or lung tissue.

Third Passage. Rabbit C.—Feb. 1, 1919. Normal leucocyte count before inoculation was 10,950, of which 5,585 were mononuclears. Temperature 39.1°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit B. Feb. 2. Both conjunctivæ injected. Leucocytes 9,300, of which 3,255 were mononuclears. Temperature 39.7°C. Feb. 3. Conjunctivitis. Leucocytes 7,350, of which 2,850 were mononuclears. Temperature 40.5°C. Killed.

Autopsy.—Lungs showed typical hemorrhagic edema and emphysema.

Aerobic Cultures.—No growth.

Fourth Passage. Rabbit D.—Feb. 3, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit C. On the next 2 days the temperature rose from 39.3° to 39.5° and 40°C. Conjunctivitis was present with a mononuclear depression of 2,455 cells on the 1st day and 1,829 on the 2nd. Feb. 5. Killed.

Autopsy.—Besides a few areas of consolidation at the base measuring 5 mm. in diameter, the lungs showed edema, patches of emphysema, and multiple punctate hemorrhages.

Aerobic Cultures.—Heart's blood, no growth; lung tissue, lower lobes Pneumococcus Type IV, upper lobes no growth.

The upper lobes were employed in the next transmission experiment.

Fifth Passage. Rabbit E.—Feb. 5, 1919. Injected intratracheally with 3 cc. of a suspension of tissue from the upper lobes of lungs of Rabbit D. During the next 2 days the temperature rose from 39.5° to 40.4° and 39.8°C. Leucopenia was noted, with a mononuclear depression from 5,377 to 2,030 on the 1st day and 3,468 on the 2nd. Feb. 7. Killed.

Autopsy.—Lungs showed the typical lesions similar to those of Rabbit B.

Aerobic Cultures.—No growth.

Sixth Passage. Rabbit F.—The effects were an exact repetition of those of Rabbit E so that it is unnecessary to give them in detail.

Aerobic Cultures.—No growth.

Seventh Passage. Rabbit G.—Feb. 9, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit F. On the 2 following days the temperature rose from 39.6° to 39.8°C. Leucopenia developed, the count diminishing from 15,640 to 12,075 and 10,725 respectively, with a mononuclear depression from 8,289 to 2,415 and 3,325 cells. Conjunctivitis. Feb. 11. Killed.

Autopsy.—Lungs showed typical lesions similar to those of Rabbits B, C, E, and F.

Aerobic Cultures.—No growth.

Eighth Passage. Rabbit H.—Feb. 11, 1919. Temperature 39.7°C. Leucocytes 12,560, of which 5,275 were mononuclears. Injected intratracheally with

3 cc. of the suspension of lung tissue from Rabbit G. Feb. 12. Leucocytes 3,250, of which 2,015 were mononuclears. Temperature 40.35°C. Feb. 13. Leucocytes 4,300, of which 2,193 were mononuclears. Temperature 40.6°C. Died.

Autopsy.—Right lung showed consolidation and fibrinous pleuritis; left lung revealed typical hemorrhagic edema with emphysema.

Aerobic Cultures.—Left lung, no growth; right lung, *Micrococcus catarrhalis.*

The left lung was employed in the next transmission experiment.

Ninth Passage. Rabbit I.—Feb. 13, 1919. Leucocytes 11,200, of which 5,488 were mononuclears. Temperature 39.2°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit H. On the next 2 days the temperature rose to 39.6° and 39.8°C., the leucocytes diminished to 8,900 and 8,675 cells, and the mononuclears to 3,382 and 2,776 respectively. Feb. 15. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Tenth Passage. Rabbit J.—Feb. 15, 1919. Injected with lung tissue from Rabbit I with similar results.

Eleventh Passage. Rabbit K.—Feb. 17, 1919. Injected similarly with lung tissue from Rabbit J. For 3 days the total leucocyte count was diminished from 10,120 to 8,320, 7,125, and 7,975, and the mononuclear count from 4,250 to 2,573, 2,494, and 1,674 respectively. Allowed to recover for experiment on immunity.

This series, therefore, was voluntarily terminated in the eleventh rabbit passage. It seems certain that it could have been continued for some time and possibly indefinitely. Besides what were regarded as typical results, this series included pneumonic infections with Pneumococcus Type IV and *Micrococcus catarrhalis* in the first, fourth, and eighth passages. Fortunately the pneumonic areas were restricted and did not lead to infection of both lungs. Hence the successive inoculation of the bacteria-free lung tissue could be continued. The death of Rabbit H of the eighth passage was the first to occur in our experiments.

PROTOCOL 4.

Patient 11.—R. N., adult male. Feb. 18, 1919. Onset sudden with chills and fever. Feb. 19. Temperature 39°C.; pulse 80. Prostration; general malaise; coryza and photophobia; unproductive cough. Physical examination showed conjunctivitis and congested pharynx; right lung, no signs; left lung, at base, few indefinite râles. Leucocytes 5,040, of which 1,764 were mononuclears. Cultures from sputum and nasopharyngeal washings yielded only *Streptococcus viridans.* Symptoms endured for 3 days, followed by an uneventful recovery.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 24 hours after the onset of the symptoms were inoculated into the lungs of Rabbit A.

First Passage. Rabbit A.—Feb. 19, 1919. Leucocytes 13,960, of which 8,794 were mononuclears. Temperature 39.3°C. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 11. Feb. 20. No change. Feb. 21. Conjunctivitis. Temperature 40.5°C. Leucocytes decreased to 6,100; mononuclears to 2,989. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Second Passage. Rabbit B.—Feb. 21, 1919. Leucocytes 12,650, of which 6,451 were mononuclears. Temperature 39.7°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit A. On the next 2 days there were fever (41.3° and 40.6°C.), leucopenia (leucocytes 5,760 and 5,775), and mononuclear depression to 2,592 and 1,098 cells. Feb. 23. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Third to Eighth Passages. Rabbits C, D, E, F, G, and H.—These rabbits developed, 24 hours after the intratracheal injection of the suspensions of lung tissue from the immediately preceding rabbit in the series, a pronounced leucopenia (in one case a decrease in leucocytes from 15,900 to 2,350), mononuclear depression (in Rabbit D, for example, from 7,632 to 940 cells), conjunctivitis, and fever (usually above 40.2°C.). The rabbits of the third, fourth, and fifth passages died 48 hours after injection; those of the sixth, seventh (Text-fig. 1, *c*), and eighth were killed in the usual manner.

Autopsy.—In all instances definite areas of consolidation were found in one or more lobes, while elsewhere the lung contained the typical areas of hemorrhage and edema accompanied with emphysema.

Aerobic Cultures.—The consolidated foci of the third, fourth, and fifth passages yielded Pneumococcus Type IV, and of the sixth, seventh, and eighth an atypical Type II pneumococcus.

Ninth Passage. Rabbit I.—Mar. 5, 1919. Leucocytes 14,225, of which 7,397 were mononuclears. Temperature 39.5°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit H. After 48 hours the temperature rose to 40°C., the leucocytes diminished to 10,225, of which 6,237 were mononuclears. This persisted for 2 days. Mar. 9. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Tenth Passage. Rabbit J.—Average leucocyte count prior to inoculation was 15,353, of which 7,587 were mononuclears. Temperature 39.6°C. Mar. 9, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit I. After 24 hours, and for the next 3 days, the temperature rose to 40°C., the leucocytes diminished to 11,400, reaching 8,800 on the 3rd day, and the mononuclears decreased to an average of 4,886 cells. Mar. 13. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

With this, the tenth rabbit passage, the series was discontinued.

This series closely reproduces the previous one. The transmission experiments were discontinued with the tenth passage and at a time when the typical effects were still being produced regularly. Several instances of fatal intercurrent infection were encountered, the pneumonia present being associated with Pneumococcus Type IV or atypical Type II. But other portions of the lungs, which were free of these and other ordinary bacteria, were suitable for the transmission experiments.

PROTOCOL 5.

Patient 17.—S. C., adult male. Apr. 10, 1919. Sudden onset at night with symptoms of languor, chills, headache, and dull pain in the back. Apr. 11. General malaise; prostration; fever; headache; no cough or rhinitis. Physical examination showed conjunctivæ injected, pharynx congested, and lungs clear (also by radiographic examination). Other organs unaffected. Leucocytes 5,875, of which 2,059 were mononuclears. Blood culture sterile. Sputum not obtainable. A throat culture yielded no Pfeiffer bacilli on oleate agar (Avery medium[12]), but a Gram-negative coccus on blood agar plates. Nasopharyngeal washings yielded this coccus and *Streptococcus viridans*. On the 3rd day of illness the temperature was normal, and an uneventful recovery followed.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 12 hours after the onset of symptoms were inoculated intratracheally into Rabbit A.

First to Third Passages. Rabbits A, B, and C.—These animals were employed for the first, second, and third passages respectively, the same methods being used as those described in the previous transmission experiments. Rabbit A died over night, Rabbit C after 2 days, and Rabbit B was killed. In all instances fever (above 40°C.) developed, with conjunctivitis and a prompt and marked leucopenia and mononuclear depression similar to those already given.

Autopsy.—The lungs revealed typical hemorrhagic edema, the hemorrhages varying from diffusely scattered small foci to large areas involving almost an entire lobe, sometimes of infarct shape. There was no definite consolidation.

Aerobic Cultures.—The lungs of the three animals yielded Type IV pneumococci.

Fourth to Fifteenth Passages.[13]—These were initiated with a filtrate, free from aerobic bacteria, of the suspension of lung tissue from Rabbit C.

In this experiment the pneumococcus present in the nasopharyngeal secretions was not suppressed during the first to third rabbit passages.

[12] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

[13] These experiments will be described in detail in a later communication.

But the effects in the animal, even though severe and fatal, bore so close a resemblance to those arising in the four previous series of rabbits that it was deemed advisable to free the material from the pneumococcus by filtration. The filtrate thus secured, free from aerobic organisms, was passed through a series of twelve animals in which the typical clinical and pathological effects were obtained.

Thus far we have dealt with cases of influenza which occurred during the epidemic of 1918–19. The next series of experiments relates to cases arising in the winter of 1920 of which three uncomplicated cases in the early stage of the affection were available for study.

PROTOCOL 6.

Patient 24.—H. J., adult female. Jan. 20, 1920. Onset sudden with chills and fever. Jan. 21. Prostration; photophobia; muscular pains; unproductive cough. Conjunctivitis and injected pharynx; lungs negative. Leucocytes 2,800, of which 336 were mononuclear. Cultures from the nasopharyngeal washings yielded numerous colonies of *Staphylococcus albus* and *Micrococcus flavus*, and a moderate number of Pfeiffer bacilli.

Animal Inoculation.—The filtered and unfiltered washings obtained 20 hours after the onset were inoculated into the lungs of three rabbits which, within 24 hours, developed conjunctivitis, fever, leucopenia, and mononuclear depression similar to those in the other instances cited. The animals were killed 48 hours after inoculation.

Autopsy.—The lungs showed the typical hemorrhagic, emphysematous, and edematous condition.

With the lungs of these rabbits another series of transmissions, consisting of three rabbits, two for the filtered material, to be described in another communication, and one for the unfiltered, was carried out. The results in each case were typical.

PROTOCOL 7.

Patient 26.—M. O., adult female. Feb. 4, 1920. Onset sudden with chills and fever. Temperature 40.1°C. Feb. 5. Fever; muscular pains; unproductive cough. Conjunctivitis; lungs clear. Leucocytes 6,000, of which 1,380 were mononuclears. Cultures from the nasopharyngeal washings yielded mainly *Staphylococcus albus* and also pneumococci, hemolytic streptococci, and occasional Pfeiffer bacilli.

Animal Inoculation.—The filtered and unfiltered nasopharyngeal washings obtained 30 hours after the onset were injected intratracheally into Rabbits A, B, and C, the last two receiving the filtrates. These animals showed the typical clinical and pathological effects, similar to those of the rabbits described in Protocols 1 and 2. No ordinary bacteria were detected in cultures or films of the lungs. The rabbits were killed 48 hours after the inoculation.

With the lungs of these animals another series of transmissions was carried out, with results which were regarded as typical. The gross appearance of the lungs of one rabbit is shown in Fig. 2. The lungs of one rabbit of the first passage of the filtered material (Rabbit C) were preserved in sterile 50 per cent glycerol for 4 months, and the lungs of Rabbit D, the second passage of the unfiltered washings, for 10 days. With these glycerolated lungs further transmissions were carried out to be described in another communication, until the experiment was voluntarily discontinued with the sixth rabbit passage. All showed the typical clinical and pathological effects, and in no instance were ordinary bacteria detected in cultures or in stained films from the lungs.

PROTOCOL 8.

Patient 27.—P. S., adult male. Feb. 9, 1920. Onset sudden with chills and fever. Feb. 10. Fever; muscular pains; sore throat; tracheal cough; headache; prostration. Lungs clear. Leucocytes 13,000, of which 10,140 were polymorphonuclears. Cultivation of nasopharyngeal washings yielded an almost pure culture of hemolytic streptococci and a few colonies of Pfeiffer bacilli. The question was raised of a complicating streptococcus sore throat in this patient.

Animal Inoculation.—The filtered and unfiltered nasopharyngeal washings obtained 20 hours after onset were injected into the lungs of five rabbits. The filtrates produced no effect; the unfiltered material caused abscess of the lung, from which hemolytic streptococci were isolated. The material was not inoculated further.

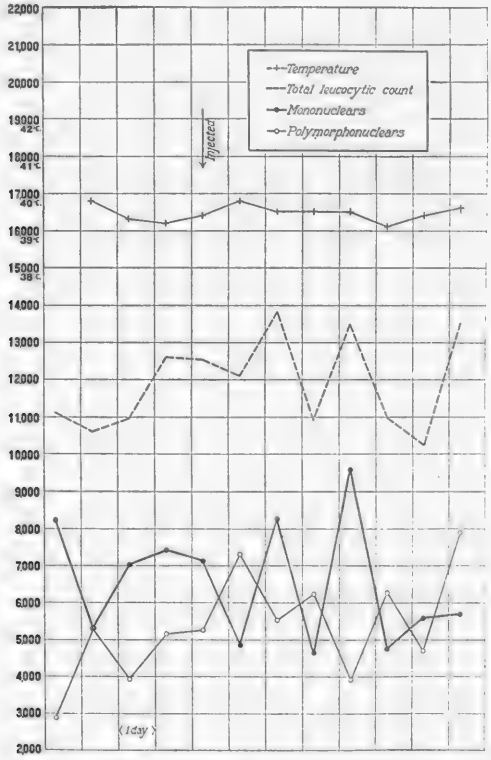
It is seen that the nasopharyngeal secretions of two of three cases of influenza during the second or 1920 epidemic gave rise in rabbits to clinical and pathological effects, independently of the presence of ordinary bacteria, similar to those obtained during the first or 1918 epidemic.

Negative Transmission Experiments.

In addition to the positive transmission experiments presented under Protocols 1 to 8, several cases of influenza, at somewhat later stages in the evolution of the disease, were studied in the same manner. The cases from the 1918 epidemic included five which were in the 3rd day of the disease when the washings were secured, four in the afebrile and convalescent stage, and two in course of a secondary pneumonia. From the 1920 epidemic one case only, on the 1st afebrile day, was studied.

The washings from the nasopharynx of these individuals were collected and injected into rabbits in the manner already described, and

in none of them was the characteristic effect on the blood count observed (Text-fig. 2). When a blood change did occur it was of the nature of a polymorphonuclear leucocytosis. The lung lesions, when any were present, consisted of lobar consolidation in which pneumococci, as a rule, were demonstrated.

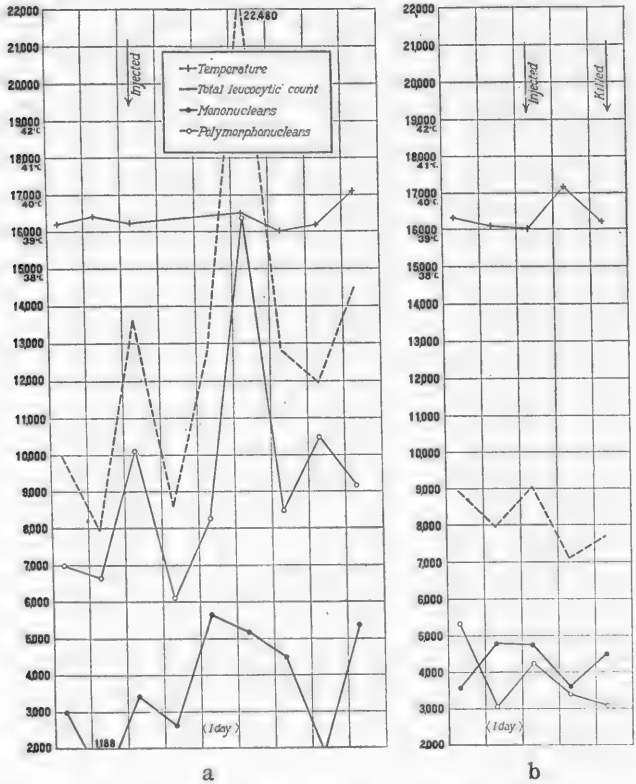


TEXT-FIG. 2. First rabbit passage of the nasopharyngeal washings from Patient 5. The washings were obtained at the beginning of the 3rd day of uncomplicated influenza. No effect on blood count and temperature.

Control Experiments (Text-Fig. 3, a and b).

The control tests consisted of the injection into the lungs of rabbits of saline solution, suspensions of normal rabbit lungs, normal rabbit serum, foreign protein, such as human ascitic fluid, bacteria of the

ordinary species,[14] including Pfeiffer's bacillus and its poison as prepared by Parker's method,[15] and finally, the nasopharyngeal secretions from fourteen persons free from influenza and tested in the epidemic



TEXT-FIG. 3, *a* and *b*. Effect on the blood count and temperature of intratracheal inoculations of control materials. (*a*) Inoculation of nasopharyngeal secretions from a normal individual free from an influenzal attack. A transient polymorphonucleosis on the 3rd day after inoculation is shown. (*b*) Inoculation of a suspension of normal rabbit lung. No effect on blood count and temperature.

[14] Control experiments with ordinary bacteria injected intratracheally will be described in another communication.

[15] Parker, J. T., *J. Immunol.*, 1919, iv, 331.

and interepidemic periods. Of the latter, seven suffered from early or later stages of coryza. The lung tissue of the inoculated rabbits was in turn reinoculated into two successive series of rabbits. None of the 55 animals inoculated with the control materials or these secretions showed the familiar clinical and pathological action; a few gave a polymorphonucleosis with frank lobar pneumonia, others a mononucleosis without lung involvement, and still others inconstant effects.

DISCUSSION.

The object of the present investigation was to determine, if possible, whether the secretions of the nasopharynx of individuals suffering from epidemic influenza exhibited on inoculation into animals any peculiarities of action or properties which would serve to distinguish them from the secretions of individuals not so affected. Obviously, the first requisite was a standard by means of which this action, or effect, could be detected. We sought one which was subject to measurement with at least a fair degree of accuracy and did not necessitate killing the inoculated animal. This criterion was found in the blood, associated with changes in the absolute and differential white blood count and correlated with the leucocytic curve in uncomplicated cases of epidemic influenza in man. A second criterion, observable after the animal had been killed, was discovered in certain hemorrhagic, edematous, and emphysematous changes in the lungs.

The next point was the determination of the relation of the changes noted in the leucocyte count and the lung structures to the ordinary bacterial flora of the nasopharynx. The fact that the changes were regularly observable, under favorable conditions, in the complete absence of ordinary bacteria in culture and in film, was regarded as particularly significant.

The deduction arrived at, therefore, as a result of the experiments carried out in rabbits was to the effect that patients with epidemic influenza, within at least the early stage of the obvious infection, carry in their nasopharyngeal secretions a substance which is not ordinary bacteria or their metabolic products. This substance when inoculated intratracheally into rabbits readily causes fever, leucocytic and particularly mononuclear cell depression, lung hemorrhage, edema, and em-

physema. Whatever this active substance is, it seems to disappear from or to diminish in the nasopharyngeal secretions of cases of epidemic influenza so as to be no longer discoverable by inoculation tests about 36 hours after the obvious symptoms of the disease have appeared, and to be absent from healthy persons and in other pathological conditions.

When implanted in the lungs of the rabbit this substance appears to increase, since it remains active through a long series of inoculations of the lung tissue in successive passages through rabbits.

The substance is readily filterable through Berkefeld filters. It is capable of surviving and apparently of multiplying in association with ordinary bacteria not only in the nasopharyngeal secretions in man but also in the lungs of rabbits, in the latter at least for a time.

When the unfiltered washings containing nasopharyngeal secretions of patients in early stages of epidemic influenza are injected intratracheally into rabbits, this substance when present exerts its peculiar action while some multiplication of ordinary bacteria (usually Pneumococcus Type IV) is going on in the lung. The successive passage of unfiltered emulsions of selected parts of the lungs, away from obviously infected and consolidated areas, leads often to rapid and complete disappearance of the ordinary bacteria and survival and possibly increase of this active substance.

No attempt will be made in this paper to define further the nature of the active substance or to relate it more accurately and specifically with the etiology of epidemic influenza.

SUMMARY.

An active substance has been detected, by the methods described, in five patients in early stages of epidemic influenza during 1918–19 and two patients in early stages of epidemic influenza during 1920. It was not detected in twelve cases of the same disease in which the onset of obvious symptoms occurred more than 36 hours before washing of the nasopharynx was carried out, nor was it found in the secretions of fourteen individuals free from the syndrome of influenza either during the epidemics or the interval between them.

With this substance a clinical and pathological condition has been induced in rabbits, affecting the blood and pulmonary structures

mainly, which could be maintained and carried through at least fifteen successive animals. For this reason, and also because of the dilution between passages, we are led to believe that we were dealing with the actual transmission of a multiplying agent rather than with a passive transference of an original active substance.

In some of the experiments secondary infections by ordinary bacteria were encountered. The relation of these microorganisms to this active substance will be dealt with fully in another communication. However, the essential effects were produced by a substance wholly unrelated to these bacteria.

The similarity that exists between the effects produced in rabbits on the blood and the lungs and those occurring in man in epidemic influenza provides a basis for further investigation on the inciting agent of epidemic influenza.

EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Gross lesions of a lung from Rabbit F, representing the sixth rabbit passage of the nasopharyngeal secretions from Patient 16. The hemorrhages, edema, and emphysema of the lung, more marked in the upper lobe, and absence of pneumonic consolidation are noteworthy. Natural size.

FIG. 2. Gross lesions of the lungs from Rabbit D, representing the second rabbit passage of the nasopharyngeal secretions from Patient 26. This case occurred in the second epidemic of 1920 and is to be compared with Fig. 1 derived from the first epidemic of 1918–19. Hemorrhages, edema, and emphysema are shown. A small area of atelectasis is seen at the inner margin of the lower right lobe. Natural size.

PLATE 6.

FIG. 3. Microscopic appearance of a section of the lung shown in Fig. 1. Edema and emphysema are present. A vessel is shown distended with blood, and mononuclears may be seen in the interalveolar tissues. × about 190.

PLATE 7.

FIG. 4. A different section of the same lung. The small discrete hemorrhages, the edema, and the cellular exudate are shown. × about 190.

FIG. 1.

FIG. 2.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. I.)

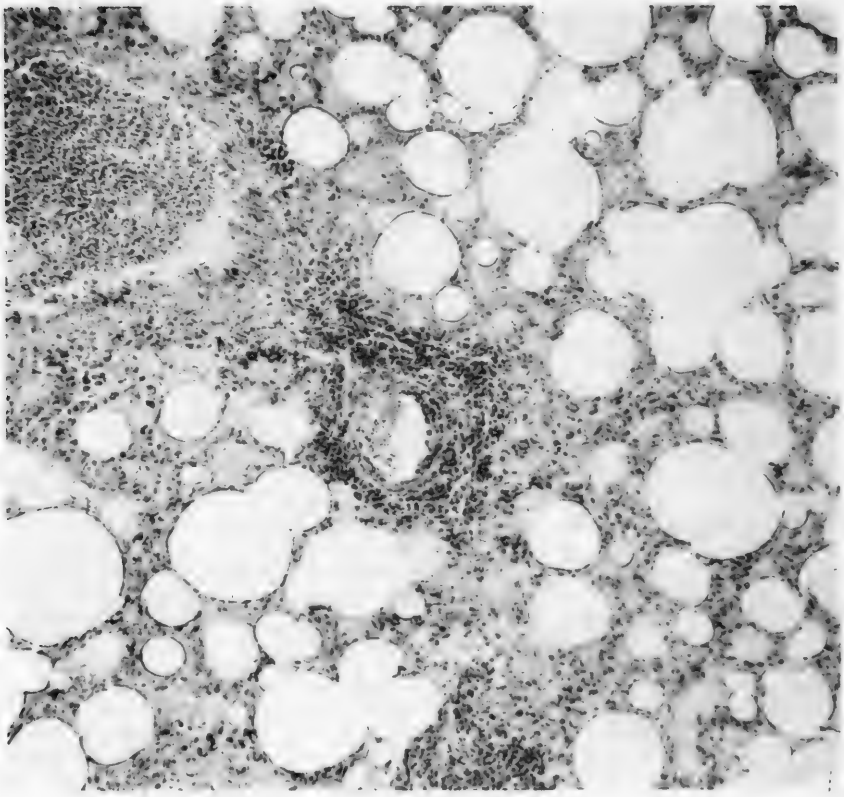


FIG. 3.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. I.)

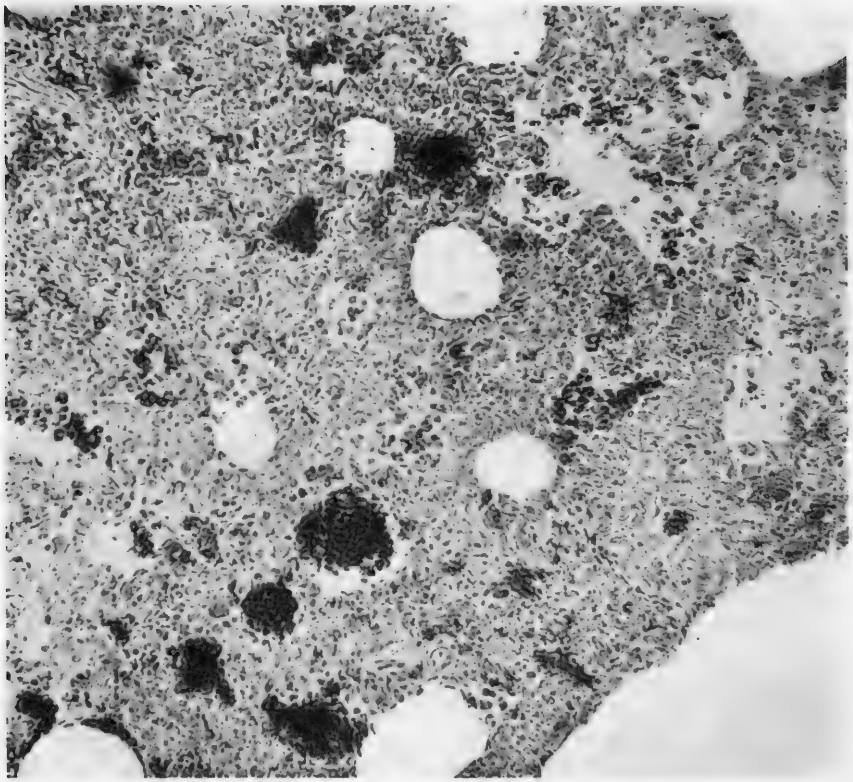


FIG. 4.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. I.)

CHILOMASTIX MESNILI AND A METHOD FOR ITS CULTURE.

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(Received for publication, July 26, 1920.)

In January, 1920, a chronic case of diarrhea due to a flagellate infection was brought to the notice of the author and upon examination the flagellate was identified as *Chilomastix mesnili*. Attempts to culture this parasitic flagellate proved successful. Observations made from time to time upon the flagellates in both the stools of the patient and the cultures, as well as data derived from a study of permanent preparations, have revealed further information regarding the biology of this organism. It is the purpose of this paper to describe this parasite and its activities and to give a method of culture for the growth of this organism in artificial medium.

Regarding its phylogeny, *Chilomastix mesnili* belongs to the family Tetramitidæ, the order Polymastigina, and the class Mastigophora.

The habitat of the parasite is the small intestine of man. It is often the cause of chronic or intermittent diarrhea. Cases of infection by *Chilomastix* in man have been reported from nearly every locality in the world. Chalmers and Pekkola[1] state that they have always found *Chilomastix* associated with other protozoa and not entirely by itself. But in the case of infection referred to above *Chilomastix* was the only protozoan parasite found. In all probability it is the cause of the patient's diarrhea. No formed stools have been passed since the discovery of the infection 2 years ago which was then thought to be caused by *Trichomonas intestinalis*. In the United States, cases of infection by *Chilomastix mesnili* are

[1] Chalmers, A. J., and Pekkola, W., *Chilomastix mesnili* (Wenyon 1910), *Ann. Trop. Med. and Parasit.*, 1918, xi, 213.

by no means rare, for Kofoid, Kornhauser, and Plate[2] found it present in 5.8 per cent of the 1,200 overseas troops which were examined at New York. Of 534 persons examined at Berkeley, California, by Kofoid and Swezy,[3] 28, or 5.3 per cent, were found infected. Many clinicians no doubt have confused *Chilomastix mesnili* with *Trichomonas intestinalis*. This mistake is due to the similarity in movement of the cytostomal flagellum of *Chilomastix mesnili* to the undulating membrane of *Trichomonas intestinalis*.

Morphology.

The cultivation of *Chilomastix mesnili* in artificial medium has made observations possible from day to day. Under these cultural conditions the flagellates grew and multiplied. Apparently the environment satisfied most of the biological requirements of the parasites. The data given here regarding the morphology and activity of these organisms are based upon direct and continued observations made upon the flagellates under cultural conditions, and also upon a study of permanent preparations made from the cultures and from the feces of the patient harboring the flagellates.

Motile Flagellate.—The location of the various organs in *Chilomastix* will be given as they are seen in a dorsal view, for it is from the dorsal view that convention or custom has always designated the right and left sides of any object.

The best descriptions of the motile or active form of *Chilomastix mesnili* have been supplied by Chalmers and Pekkola[1] and more recently by Kofoid and Swezy.[3] The descriptions given by these two groups of workers differ in some respects; that of Kofoid and Swezy,[3] however, is the more valuable because details of morphology are given which were previously unknown. The description given here corroborates that of Kofoid and Swezy[3] in nearly all details. The differences of interpretation and interpolation of new data regarding the morphology and the activities of the flagellates have come as a result of continued study of the organisms in cultures over a period

[2] Kofoid, C. A., Kornhauser, S. I., and Plate, J. T., Intestinal parasites in overseas and home service troops of the U. S. Army, *J. Am. Med. Assn.*, 1919, lxxii, 1721.

[3] Kofoid, C. A., and Swezy, O., On the morphology and mitosis of *Chilomastix mesnili* (Wenyon), *Univ. California Pub., Zool.*, 1920, xx, 117.

of 4 months. The cultures were inoculated on January 27, 1920, and were kept alive by subculturing until June 15, 1920, when a mishap to the heating apparatus of the incubator caused it to reach 80°C., which resulted in the death of the organisms.

The usual shape of the body of *Chilomastix mesnili* is pyriform with a rounded anterior end and a pointed posterior end terminating in a spine-like process. The latter structure is composed almost entirely of hyaline periplast. The sides begin to taper posteriorly at the middle of the body. This tapering is not uniform for both sides but varied, depending upon the particular state of locomotion the flagellate may be in at any given moment. It is not uncommon to find forms which have the ventrum flattened, and the dorsum arched, so that in profile they resemble a side view of *Giardia intestinalis*. When these particular flattened forms were encountered, the posterior spine appeared as a posterior terminal extension of the ventrum, while in other forms the spine is terminal but more axially situated.

In the stools, the active flagellates have been seen to assume the shape of a sphere, and the spine-like protuberance has been withdrawn by contraction. On two occasions twisted forms have been seen similar to those described by Wenyon and O'Connor,[4] Chalmers and Pekkola,[1] and Kofoid and Swezy.[3] The twisted forms have not been encountered in the cultures. Fortunately, it was possible to observe these atypical flagellates for a considerable length of time and evidence was obtained which may explain their twisted condition.

The twisted state of the flagellate appears to be a temporary condition, at least as far as the flagellates in this particular case are concerned.

Wenyon[5] did not note this twisted condition in his original description of *Chilomastix mesnili* from man, and it was not until later that he and O'Connor[4] described it. Chalmers and Pekkola[1] spoke of this twisted condition as due to "a dorsal twisting of the antero-ventral part, while the posterior part of the body is not affected. This twist naturally gives rise to the appearance of lines and

[4] Wenyon, C. M., and O'Connor, F. W., Human intestinal protozoa in the Near East, London, 1917.

[5] Wenyon, C. M., A new flagellate (*Macrostoma mesnili n. sp.*) from the human intestine with some remarks on the supposed cysts of *Trichomonas*, *Parasit.*, 1910, iii, 210.

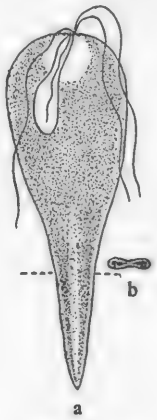
grooves running more or less diagonally backwards across the body." Kofoid and Swezy[3] found that this twisted condition, resulting in a diagonal groove, characterized most of the active forms of *Chilomastix mesnili* and therefore concluded that the groove was a permanent structure. They also believed that in the forms where the groove was not in evidence, it had temporarily disappeared due to the contraction of the body.

Since January, 1920, the author has received fecal specimens once or twice each week from the patient infected with *Chilomastix mesnili*. As has been said before, on only two occasions were the twisted forms encountered. On these occasions it was also found that the flagellates were definitely changed in shape. The anterior end was rounded as usual but the posterior half of the body was broadly flattened from side to side. A dorsal view showed the body almost truly pyriform with uniformly tapering sides (Text-fig. 1). A lateral view resembled the side view of a tadpole, for the posterior end possessed a broad flattened tail instead of a pointed spine (Text-fig. 2, *a*). On closer observation it was seen that the tail contained two rib-like extensions of the granular endoplasm running out into the hyaline periplast of the ectoplasm. These two ribs of endoplasm were united anteriorly where they joined the mass of endoplasm of the anterior portion of the body, but they began to diverge as they proceeded posteriorly and became separated by a thin membrane-like structure made only of the periplast. The area of the tail, constituted only of the membrane of periplast between the two ribs of endoplasm, was thinner than the adjacent areas occupied by the endoplasmic ribs, which were also surrounded by periplast. This condition resulted in the formation of a straight groove on each side of the body between these extensions of the endoplasm (Text-fig. 2, *b*).

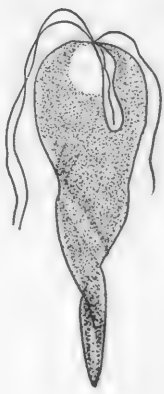
These forms, in addition to being laterally flattened, assumed the twisted condition both when at rest and during locomotion. They possessed definite grooves running diagonally and posteriorly from the left to the right side (Text-fig. 3). To all appearances these flagellates were similar to the twisted forms described by Wenyon and O'Connor,[4] Chalmers and Pekkola,[1] and somewhat like those described by Kofoid and Swezy.[3] In view of the presence of the laterally flattened forms which showed grooves in the posterior region, but were not twisted, it seemed plausible that the grooves of the

twisted forms were identical with those of the flagellates which were not twisted, and that this state was a temporary condition brought about by some factor which accounted for the change in form of the flagellates. The flagellates which were not twisted but were flattened may either have recovered from the twisted condition or not as yet become so transformed. The temporary nature of the twisted condition of the flagellates of this particular case of infection seems all the more substantiated since it was never observed in the flagellates which have been growing and reproducing in cultures for over 4 months.

TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 1. *Chilomastix mesnili*, dorsal view, unstained. Posterior region compressed. × 3,300.

TEXT-FIG. 2. *Chilomastix mesnili*, unstained. (*a*) Lateral view showing compressed tail; (*b*) cross-section of tail to show grooves. × 3,300.

TEXT-FIG. 3. *Chilomastix mesnili*, dorsal view, unstained. Twisted form showing diagonal groove. × 3,300.

The motile organisms observed in this case measured 3 to 19 microns in length and 2 to 9 microns in width. Most forms in the stool were 8 to 14 microns in length. The flagellates possessed three anterior flagella and a cytostomal flagellum situated in the oral cavity, or cytostome. In a dorsal view (Text-fig. 4) the cytostome is located on the ventrum and the right side, extending backward a distance of one-third to one-half the length of the body. The nucleus

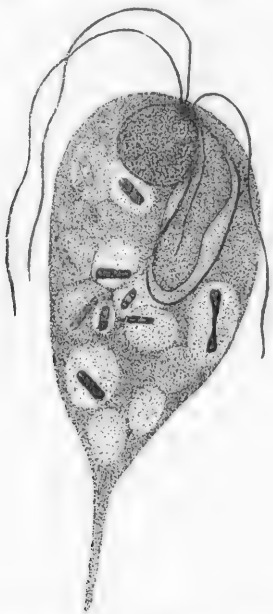
is round, and located near the anterior border of the body, to the left of the cytostome and dorsal to it. In unstained specimens it appears somewhat refractile. The cytoplasm is granular and generally contains large vacuoles, in many of which was a bacterial cell which had been previously ingested as food. At the times when binary fission is in process a tendency probably exists on the part of the flagellate to desist from eating, for in many of the forms undergoing division no vacuoles containing bacteria were seen.

In locomotion, *Chilomastix mesnili* is propelled forward and spirally in a jerky manner by the action of its flagella. The two left anterior flagella, as Kofoid and Swezy[3] have pointed out, beat backward and against the left side of the body, and the other anterior flagellum beats backward against the right side of the body. The motion of these flagella is undulatory, the ripples starting at the point of origin of the anterior flagella and proceeding posteriorly. These waves of contraction diminish as they progress distally along each flagellum.

The cytostomal flagellum lies within the cytostome and forms an undulating membrane by adhering to the inner portion of the cytoplasmic lip or membrane which is also within the cytostome (Text-fig. 4). This flagellum is probably not involved in any purpose of locomotion but functions chiefly as a means of obtaining food. Gäbel[6] and Chalmers and Pekkola[1] noted that the flagellum may not always form an undulating membrane but may act as a free flagellum. In the writer's observations upon living forms thus far the flagellum has not been seen as a free whip but always combined with the thin cytoplasmic lip to form an undulating membrane. In the flagellates more or less quiescent, the action of this cytostomal undulating membrane was easily observed. The undulating waves of contraction began at the anterior end of the cytostome at the place of origin of the cytostomal flagellum and proceeded posteriorly to the end of the cytostome. The initial wave of contraction of the undulating membrane was very large, for at times the membrane was pushed outward from the cytostome 3 to 4 microns. The amplitude of this first wave of contraction diminished as the wave progressed posteriorly. The action of the undulating membrane had the effect of

[6] Gäbel, M., Zur Pathogenität der Flagellaten, *Arch. Protistenk.*, 1914, xxxiv, 1.

reaching out into the surrounding medium and literally scooping the bacteria toward the cytostome. In this way food was brought in reach of the cytostome. The anterior flagellum which beat backward against the right side was very close to the cytostome and aided materially in setting up a current in the medium which helped to bring the food in proximity to the cytostome



TEXT-FIG. 4. *Chilomastix mesnili*, dorsal view, stained. Taken from culture. × 4,400.

The cytostome has had holdfast properties attributed to it by Kofoid and Swezy.[3] No doubt because of its size and morphology it may serve such a purpose, but in the study of the flagellates in the cultures, as well as in fecal specimens, the posterior spine or protuberance was used for this purpose almost entirely.

When the flagellate was feeding, it inserted this posterior spine into a mass of bacteria, while its body remained free from any encumbrance. In this position the flagellate anchored itself and proceeded

to rotate, using the posterior spine as the holdfast organ. On such occasions one could watch the undulating membrane as it wafted bacteria into the cytostome. Not all the bacteria which were taken into the cytostome were ingested, for many of them could be seen escaping in a current in the medium directed away from the cytostome. It is very likely that the flagellate exercised a selective sense in the choice of its food.

The finer details of morphology have been described by Kofoid and Swezy.[3] They have shown *Chilomastix mesnili* to possess an elaborate neuromotor apparatus. The author has been able to verify the finding of the structures described by these workers, but because the finer morphological structures which only become apparent in properly prepared, stained specimens have received a full description by the above investigators, brief reference is made to them in this paper.

The nucleus is round and possesses a distinct membrane (Text-fig. 4). It is located near the anterior border of the body to the left of, and dorsal to the cytostome. The chromatin may be distributed in a variety of ways, but it is often in the form of two terminal masses lodged against the nuclear membrane with a small eccentrically placed karyosome, or broken up into chromidia distributed diffusely throughout the nucleus. The chromatin was also resolved into a definite spireme strand, which in some forms had later segmented to form chromosomes.

A very small centrosome is located at the anterior pole of the nucleus (Text-fig. 4). It is often concealed by a larger chromatin-staining mass, the blepharoplast complex. As Chalmers and Pekkola,[1] and Kofoid and Swezy[3] have shown, there are three blepharoplasts which are only seen as separate granules in well decolorized preparations. The primary blepharoplast lies closest and anterior to the nucleus. It gives origin to the pair of anterior flagella which beat backward against the left side of the body. The secondary blepharoplast lies farther to the right side and gives rise to the right anterior flagellum. Kofoid and Swezy[3] found that this secondary blepharoplast also gives rise to the parastyle, a slender, slightly curved rod, which extends posteriorly in the left wall of the cytostome. The tertiary blepharoplast lies at the anterior end of the

cytostome and gives rise to the cytostomal flagellum, the peristomal fiber, and the parabasal. All the blepharoplasts are joined together in series with the centrosome and often with the anterior terminal mass of chromatin of the nucleus by fine rhizoplasts. In many forms the primary and secondary blepharoplasts are fused into a single large granule located against the nuclear membrane at the anterior pole of the nucleus (Text-fig. 4). This double blepharoplast by virtue of its position often obscures the centrosome.

The peristomal fiber borders the inner free edge of the membranous lip of the cytostome. It is a fine fibril originating from the tertiary blepharoplast. The cytostomal flagellum when in action appears to adhere to the inner portion of this loose membranous lip to form an undulating membrane within the cytostome, or oral pouch. That this is the case is evidenced by the location of the cytostomal flagellum lying along the side of, and adhering to the inner portion of this lip in many stained specimens. Furthermore, in moribund flagellates it is the inner portion of the lip which is seen to push out for a great distance to begin a wave of contraction which lessens and dies out as the contraction moves posteriorly. In other cases the cytostomal flagellum may be seen to lie on the floor of the cytostome (Text-fig. 4) or at times just under the inner portion of the peristomal fiber.

The parabasal is a large chromatin-staining rod extending from the tertiary blepharoplast along the right side and around the posterior end of the cytostome. In some forms it appears to surround completely the cytostome. However, Kofoid and Swezy[3] found that in such cases, if the preparations have been well destained, this rod is not continuous but is composed of two elements, the parabasal, extending from the tertiary blepharoplast along the right side and around the posterior end of the cytostome, and another rod, the parastyle, arising from the secondary blepharoplast and extending along the left side of the cytostome to within a very short distance of the posterior end of the parabasal. Chalmers and Pekkola[1] believed the parabasal was a continuous rod arising from the secondary blepharoplast and returning to it after it had run a circuitous course around the cytostome.

Kofoid and Swezy[3] have shown the homology which exists between the peristomal fiber and parastyle of *Chilomastix mesnili* with the

peristomal fiber and the right axostyle of the right half of *Giardia*. There is good morphological evidence to draw a homology between the peristomal fibers of *Chilomastix* and *Giardia*, and likewise between the parastyle of the former with the right axostyle of the latter organism, but there remains the problem of finding a homologue in *Giardia* for the parabasal of *Chilomastix*. It is more than likely that the parabasals of *Giardia* differ so much in structure and chemical constitution that they cannot be considered homologues of the parabasal of *Chilomastix*. On the other hand, Chalmers and Pekkola[1] showed the parabasal of *Chilomastix* to be homologous to the parabasal of *Trichomonas*, since in both these organisms this rod has the same morphological relation to the undulating membrane. In view of these relations existing between the structures of *Chilomastix*, *Trichomonas*, and *Giardia*, *Chilomastix* may be said to occupy a position midway between the trichomonad flagellates and members of the genus *Giardia*. *Chilomastix* may have resulted as an offshoot from the trichomonad flagellates, and, as Kofoid and Swezy[3] have pointed out, it may be the form from which *Giardia* and other Hexamitidæ have originated.

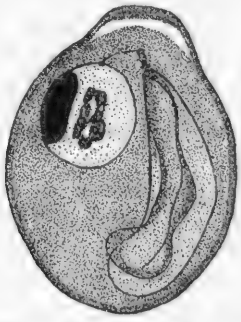
Cysts.—The cysts of *Chilomastix mesnili* are usually found in varying numbers along with the active flagellates. However, they may be entirely absent from a fecal specimen containing an abundance of motile organisms. This fact is evidence of encystment being an irregular if not a cyclic process dependent perhaps on many causes. Wenyon and O'Connor,[4] in the case of a patient undergoing treatment for *Entamœba histolytica*, record the absence of the cysts of *Chilomastix* for 20 days; the flagellates, however, were present on 13 days of this period. In the fecal samples received for examination once or twice each week since January, 1920, the cysts have been absent on only a few occasions.

The cysts are usually somewhat lemon-shaped, with one end rounded and the other end terminating in a slight protuberance (Text-fig. 5). Occasionally they are ovoid and lack the terminal process. In size, the cysts measure 4.5 to 6 microns in width and 7.6 to 9 microns in length. Wenyon and O'Connor[4] record cysts with a length of 10 microns.

The wall of the cyst is transparent and of uniform thickness except in the vicinity of the terminal elevation where it is thicker than at other places.

The cytoplasm within the wall of unstained cysts is refractile and structureless except for the presence of a few bright spots. With favorable lighting facilities for the microscope, a dull brownish area can be seen which is the nucleus. There is a space between the cytoplasm and the cyst wall immediately back of the terminal protuberance.

In stained specimens, the cytoplasm appears to be very finely granulated. At one side toward the anterior end is a large round or ovoid nucleus. The chromatin is often distributed in the form of one large karyosome with accessory chromatin matter, or as a central



TEXT-FIG. 5. *Chilomastix mesnili* cyst, stained. × 4,400.

karyosome with one or two terminal masses, or broken up into chromosomes. Kofoid and Swezy[3] have described in detail the mitosis which takes place within the cyst. These investigators were the first to find cysts containing two nuclei and two sets of neuromotor apparatus. The karyosome of the nucleus is often connected by a rhizoplast with the centrosome on the nuclear membrane at the anterior pole of the nucleus. The centrosome is in turn connected with the blepharoplasts by fine rhizoplasts (Text-fig. 5). In many cysts, as in the case of the motile flagellates, the distinct entity of all three blepharoplasts cannot be made out, only two masses being apparent. The primary and secondary blepharoplasts have become united into a single large granule. The tertiary blepharoplast remains separate at the anterior end of the cytostome.

The entire structure of the neuromotor apparatus of the motile organism is preserved and carried over into the cyst. It is possible in properly stained specimens to locate the peristomal fiber, the parabasal, the parastyle, and the cytostomal flagellum. In many cysts, however, some members of this fibrillar apparatus are not discernible. The failure to see these structures may be due to imperfect fixation and decolorization after staining or the structures may have actually disintegrated.

Method of Culture.

Medium.—Less difficulty was experienced in culturing *Chilomastix mesnili* in artificial medium than was anticipated. There were only two fundamental substances used in the preparation of the culture medium; namely, blood serum and a physiological solution. Three kinds of blood serum, horse, sheep, and human, were combined with varying proportions of either Ringer's or Locke's solution. In the attempt to find a suitable medium, the proportions of blood serum and the physiological solutions were varied from equal parts of both to one part of blood serum and two to seven parts of the Ringer's or Locke's solution. But after trying out these various combinations, it was found that the flagellates grew best and multiplied in number in a medium containing the proportion of one part of human blood serum to four parts of Locke's solution. There were limited growth and reproduction in a medium made from one part of either horse or sheep serum to four parts of Locke's or Ringer's solution. But a culture medium containing Ringer's solution did not give as satisfactory results as one containing Locke's solution, and human serum promoted better growth than either the sheep or horse serum.

The human serum[7] comprised the unused portions of the samples sent in to the laboratory for the Wassermann reaction. Both negative and positive sera were used, and they were equally satisfactory.

[7] This serum was received in a sterile condition from the Department of Bacteriology of the Baltimore City Board of Health through the kindness of Dr. William R. Stokes.

The Locke's solution was made up as follows:

Sodium chloride	0.9	gm.
Calcium chloride	0.024	"
Potassium chloride	0.042	"
Sodium bicarbonate	0.02	"
Distilled H_2O	100.00	cc.

To this solution 0.25 gm. of dextrose was added, and it was then sterilized. After the sterilization of the Locke's solution the human serum was mixed with it in the proportion given above, care being taken to prevent any contamination of the resulting medium. The culture medium was distributed in 5 cc. amounts in small test-tubes and these were incubated at 37°C. over night and examined the next morning. The tubes which showed any evidence of bacterial growth were discarded. The medium generally had a clear cream-colored appearance unless blood cells were present in the blood serum, in which case the medium was reddish in color until the blood cells sank to the bottom of the tube. If in the samples of blood a slight amount of hemolysis had taken place, the serum was tinted red by the hemoglobin. Culture medium containing either red blood cells or hemoglobin as the result of the hemolysis of the cells did not prevent the growth and propagation of the flagellates but at times it appeared more favorable for their growth than culture medium devoid of these substances.

The reaction of several batches of the culture medium varied from neutral to 0.4 per cent alkaline to phenolphthalein. A reaction of 0.2 per cent alkaline usually resulted from the mixture of the human blood serum and Locke's solution.

Technique.—It was found advisable to wash a small portion of the stool containing the flagellates before inoculating the culture medium, in order to get rid of a superabundance of bacteria, fecal debris, and products of chemical decomposition which in the beginning might prove inhibitory to the growth of the organisms in artificial medium. Accordingly a loop of fecal material containing numerous flagellates was washed by stirring it in with about 2 cc. of warm sterile normal saline solution and then taking a couple of drops of this suspension containing the flagellates and mixing it with another small amount of normal saline solution. This procedure was followed a third time

if it was found that the second resulting suspension was very turbid from the presence of much fecal detritus and numerous bacteria. A couple of drops from each of the above suspensions were inoculated into each of several tubes of culture medium and incubated at 37°C.

On the following day the tubes were examined. Some showed an absence of flagellates; others showed the presence of *Chilomastix* but in small numbers. Transplants were made from the positive cultures by transposing several loops of medium containing the flagellates. On the 2nd day after the original inoculation the positive tubes showed an increase in the number of organisms. Their number was increased even more the following day, at which time more transplants were made. The first inoculation was begun on January 27, 1920, and transplants were made at various intervals. The flagellates were kept in this artificial medium from the above date to June 15, 1920, when an accident to the heating device of the incubator caused the temperature to rise to 80°C., which killed the flagellates.

Other attempts to grow *Chilomastix* taken from the feces in the Locke-human serum medium have proved successful and have demonstrated the favorableness of this artificial medium as a habitat for these parasitic flagellates of man.

Growth in Cultures.

Period of Lag.—The life of a culture, as denoted by the presence of living flagellates, varied in length from 2 to 10 days. The average length of life for each culture was about 6 to 8 days.

The transplants from old to new cultures were usually made in the morning. Such inoculations into new cultures always carried over numerous flagellates and bacteria from the old culture. Little or no growth was noticeable during the early hours of incubation of the new cultures. A period of lag, similar, perhaps, except in length, to the lag phase in fresh cultures of bacteria, apparently characterized the growth of the flagellates during the early hours of the life of a culture. The evidence for a lag phase in the growth of these protozoa in cultures was obtained from the observation of cultures at frequent intervals during the first 22 hours of incubation.

A culture of *Chilomastix* which was transplanted at 9 a.m. and kept in the incubator at 37°C. was observed hourly throughout the same day and night up to 7 a.m. of the following day. Likewise vaseline preparations on depression glass slides were inoculated at the same time as the above culture and observed at the same intervals. Several loops of the culture were withdrawn for observation at each interval of an hour. The vaseline preparations permitted observation of their entire contents, since these preparations had been made upon glass slides which were easily manipulated upon the stage of the microscope.

The first signs of dividing organisms were noticed at 10 p.m. and the flagellates were scarce in number. At midnight, 15 hours after inoculation, the dividing forms were encountered more frequently, and dividing flagellates were in evidence at all subsequent examinations. The number of dividing forms seen during any one observation never exceeded six, but in view of the small volume of several loops of medium taken for each observation, the rate of reproduction was high. The period of lag, in the case of this particular culture, was from 9 a.m. until 10 p.m., or 13 hours in length. During this period of lag it is more than probable that multiplication of the organisms was going on, but the organisms were so few in number that they were missed in the small sample of medium taken for observation. In view of this circumstance it cannot be definitely concluded that the period of lag is 13 hours in length, for with more experiments it may be found to vary considerably.

Presence of Bacteria.—During the 1st day in the life of a culture, much gas was produced by the bacteria. A slight scum appeared on the surface of the medium and small bacterial clumps were present at the bottom of the culture. The scum became thicker and the precipitation of bacteria forming clumps became greater during the subsequent days of growth in the cultures.

Most of the bacteria present in the cultures were identified and were chiefly members of the colon-typhoid group. The bacteria identified were *Bacillus coli communis*, *Bacillus coli communior*, *Bacterium aerogenes*, *Bacillus alkaligenes*, and *Bacillus proteus*. *Bacillus subtilis* was found in a few cultures; whether or not it promoted or inhibited the growth of the protozoa was not determined. It

was not present in early cultures and no transplants were made from cultures containing this bacillus.

At the death of a culture, when all the flagellates had disappeared, the reaction of the medium had been changed by the activities of the bacteria. The reaction at the end was always more strongly alkaline. One would expect that the presence of *Bacillus coli*, *Bacterium aerogenes*, and *Bacillus proteus* would produce a sufficient quantity of acid by the fermentation of the dextrose in the medium to turn the alkaline reaction of the medium in the beginning, to acid, 8 days later. The gas present in the cultures during the first few days after inoculation was the result of the fermentative action of *Bacillus coli*, *Bacillus proteus*, and *Bacterium aerogenes*. During this time it is probable, for it was not determined, that the reaction might have been slightly acid, but the effect in such a case was only temporary as the reaction had become alkaline in the end. The increase in alkalinity might have been due to the presence of *Bacillus alkaligenes*, for it was found that this organism was the most predominant of all the bacteria, as evidenced from the number of colonies on an agar plate planted from a culture in which all the flagellates had disappeared.

Most of the flagellates were found feeding upon the bacterial clumps at the bottom of the culture. They were so numerous on the 3rd or 4th day after inoculation of the cultures that they often appeared as a compact mass, with their bodies touching each other and showing little or no progressive locomotion. The presence of most of the flagellates at the bottom of the culture would indicate their preference for a medium in which the oxygen content was somewhat reduced.

Size of Flagellates.—The size of the flagellates in the cultures ranged from 3 to 18 microns in length and from 2 to 9 microns in width. During the first 3 or 4 days when the flagellates were numerous, there was a great number of small forms measuring from 3 to 8 microns in length. The small size of these forms was undoubtedly due to the rapid rate of reproduction which took place during the 1st day especially, and later, during the 2nd, 3rd, and 4th days. This rapid proliferation on the part of the flagellates did not permit of the organisms reaching their full growth previous to another division. Many of the small forms were probably the products of multiple fission. Near

the end of the life of the culture when the number of flagellates was small, the organisms were apparently normal in size; *i.e.*, about 8 to 14 microns in length.

The difference in the average size of the flagellates in the cultures and those obtained directly from the feces was due apparently to a more rapid rate of multiplication in the culture than in the intestine of man. The factors which might have caused this great rate of reproduction in the cultures had perhaps a better opportunity of influencing the growth of the protozoa in the test-tube than in the intestine of man, since they were concentrated into a smaller area. And furthermore, they accumulated more rapidly in the cultures than in the human intestine where digestion was accomplishing changes in the intestinal medium of the organisms.

Cysts.—Thus far, no cysts have been found in any of the cultures. Whether encystment was inhibited by the lack or presence of certain substances in the medium was not ascertained. Skatole has been known to cause encystment of amebæ, but as yet no experiments have been made with the use of this product of protein decomposition to induce the encystment of *Chilomastix*.

Rapidity of Growth.—A detailed study made on five different cultures of *Chilomastix* furnished data as regards the growth and multiplication of the flagellates. The activities of the flagellates in these cultures were not unlike those in all the cultures made of *Chilomastix*.

The number of flagellates per cubic centimeter of culture medium was computed for each day during the lifetime of the five cultures. The method of estimating the number of flagellates per cubic centimeter was as follows: Each culture was shaken vigorously for 1 minute previous to examination in order to distribute the organisms uniformly throughout the medium. Only a sufficient amount of the medium containing the flagellates was placed in the counting chamber of a hemacytometer to prevent an overflow into the moats of the chamber. Immediately before placing the flagellates in the counting chamber, a minute drop of saturated corrosive sublimate solution was placed on the floor of the counting chamber and mixed with the culture medium as both were drawn into the chamber by capillary attraction. The drop of saturated corrosive sublimate solution was used to kill the flagellates, thus preventing their moving

about over the ruled area and avoiding more than one count. This minute drop of corrosive sublimate solution increased the percentage of error to some extent, yet the total count of organisms per cubic centimeter as determined by this method can be said to be fairly representative of the actual number. All the flagellates were counted which were found lying within the confines of the total ruled area of the counting chamber and by simple calculation the number of flagellates in a cubic centimeter was computed.

Each of the five cultures containing 5 cc. of medium with a reaction of 0.1 per cent alkaline to phenolphthalein was inoculated on May 19, 1920. Each culture was inoculated with three loops of medium from another culture having about 200,000 organisms per cubic centimeter. In a representative loopful, about 250 flagellates were counted, hence it follows that each culture received about 750 organisms, or about 150 flagellates for each cubic centimeter of the new culture.

The number of organisms per cubic centimeter from day to day for all five cultures is given in Table I. The per cent increase in the number of flagellates is also given for each day.

On the following day, May 20, there was evidence of the great fertility of these flagellates in the great increase in their number. Culture 3, which contained such a scattering of organisms that they were too scarce to be counted with the hemacytometer, was an exception; but it behaved similarly on the following day. This latter culture showed no apparent increase in the number of flagellates and in all probability contained few more than the original number inoculated. Proliferation had been inhibited or delayed for a period of 1 day. The remarkable fecundity of these flagellates is surprising when one notices in Culture 2 an increase in the number of flagellates from 150 per cubic centimeter to 26,666 per cubic centimeter, an actual increase over the number of May 19 of 17,677.3 per cent. Cultures 1, 4, and 5 also showed remarkable increases in the number of flagellates, Culture 4 being the lowest with an increase of 5,405 flagellates, or 3,603.3 per cent, over the number of the day before. As has been said, the greatest multiplication takes place during the 1st day.

On the next day, May 21, the per cent of increase in the number of flagellates had fallen for Cultures 1, 2, 4, and 5, although the per cent increase for Culture 4 was about the same as that of May 20. Culture 3 showed a large increase from about 150 to 11,111 flagellates per cubic centimeter, an increase of 7,307.3 per cent. Culture 5 had the largest number of organisms per cubic centimeter, which was about 277,777. Culture 2 followed closely.

TABLE I.

Date.	Culture No 1		2		3		4		5	
	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.
1920										
May 20	15,555	<10,270.0	26,666	<17,677.3	Few.		5,555	<3,603.3	20,000	<13,233.3
" 21	150,000	<864.3	253,000	<848.7	11,111	<7,307.3	204,444	<3,580.3	277,777	<1,288.8
" 22	255,555	<70.3	702,222	<177.5	293,333	<2,540.0	437,777	<114.1	725,555	<161.2
" 23	280,000	<9.5	300,000	>57.2	368,000	<25.4	524,000	<19.7	365,555	>49.6
" 24	232,222	>17.0	208,888	>30.3	357,777	>2.7	472,222	>9.8	401,111	<9.7
" 25	216,666	>6.6	153,333	>26.5	255,555	>28.5	327,777	>30.5	Dead.	
" 26	24,444	>88.7	Dead.		23,333	>90.8	Dead.			
" 27	Dead.				Dead.					

Cultures inoculated May 19, 1920. Number of flagellates per cubic centimeter and per cent increase recorded for each day. < indicates increase; >, decrease.

On May 22, the per cent increase in the number of organisms dropped again for all the cultures including Culture 3. 3 days after inoculation the maximum number of flagellates per cubic centimeter was reached in Cultures 2 and 5 which was 702,222 and 725,555 respectively. Culture 3 showed a much larger number than the day before, increasing from 11,111 to 293,333 flagellates per cubic centimeter, an increase of 2,540 per cent.

On May 23, Cultures 2 and 5, having reached the maximum number of flagellates per cubic centimeter on the previous day, now showed a marked decrease per cubic centimeter of 402,222 flagellates (57.2 per cent) for Culture 2, and 360,000 flagellates (49.6 per cent) for Culture 5. 4 days after inoculation, Cultures 1, 3, and 4 contained the largest number of flagellates per cubic centimeter, although the number of flagellates for each culture was considerably less than the number attained in Cultures 2 and 5 on the day before. Also the per cent increase in the number of organisms for Cultures 1, 3, and 4 was comparatively low.

On the next day, May 24, Cultures 1, 3, and 4 first began to show a decrease in the number of flagellates, but the decrease, ranging from 2.7 to 17 per cent was much smaller than the initial per cent decrease of Cultures 2 and 5 on the previous day. And on this day, Culture 2 continued to decline in its number of flagellates but Culture 5 showed evidence of some increase, for it gained from 365,555 to 401,111 flagellates per cubic centimeter. Nevertheless, it still showed a 44.7 per cent reduction from the maximum number of organisms of May 22.

On May 25, all the cultures showed a greater falling off in the number of flagellates from the day before, and Culture 5 no longer contained flagellates. A drop of 401,111 flagellates per cubic centimeter to none at all had occurred in the 24 hour interval between examinations.

On May 26, Cultures 2 and 4 were also found to contain no flagellates, while Cultures 1 and 3 had suffered a tremendous loss. The decrease was 88.7 and 90.8 per cent, respectively. These large decreases in the number of flagellates and the per cent were forewarning that the organisms would not be found alive much longer, and on the next day, May 27, all had disappeared.

From the study of these five cultures, it was seen, as it had been noticed in all previous cultures, that the period of the greatest rate of proliferation took place during the 1st day and that the greatest number of flagellates was to be found in the cultures on the 3rd or 4th day. Rarely a second phase of multiplication occurred, and when present it usually came on the 5th day when a marked decrease in the number of organisms had already taken place on the day before. Culture 5 showed evidence of this behavior. The rate of increase in the number of flagellates decreased after the 1st day, becoming less and less up to the 3rd or 4th day. Then a decrease in the number of

flagellates occurred, which ended in their disappearance on the 6th, 7th, or 8th day. The rate of increase and decrease in the number of organisms varied for each culture, although they were somewhat similar, in that one could expect an increase during the early days in the life of a culture and a decrease after the 3rd or 4th day. In some instances the rates in per cent of increase and decrease were similar for different cultures. The death and disappearance of all the organisms on the last day were sudden, thousands of flagellates per cubic centimeter being destroyed within a single day.

Food.—The food of *Chilomastix* was comprised chiefly of bacteria although the mineral salts of the Locke's solution, the proteins of the blood serum, and the sugar may also have served to some extent as food and a source of energy. But one cannot observe the flagellates using the latter substances as food as they do the bacteria in the medium. When the bacteria are ingested they may be seen within the food vacuoles of the cytoplasm. The process of ingestion has been described above.

It is probable that the presence of the bacteria whether they were for the purpose of food or not may explain in part some of the more important questions concerned with the life of the flagellates in the cultures.

Fertility and Death of the Flagellates.—No definite evidence has been uncovered as yet to explain the great fertility of the flagellates during the 1st day in a new culture but certain theoretical conclusions warrant consideration. It has already been said that a period of lag preceded the period of excessive and rapid multiplication. This lag period, as yet, has not had its length of time determined, although the first dividing flagellates were found 13 hours after the inoculation of the cultures.

In the growth of bacteria a period of lag has been known for some time past. Lately, Salter[8] working with *Bacillus coli communis* showed that the length of this period was from 3 to 8 hours, depending upon the medium used and the age of the original culture from which subcultures were made. It is probable that the period of lag of

[8] Salter, R. C., Observations on the rate of growth of *B. coli*, *J. Infect. Dis.*, 1919, xxiv, 260.

Bacillus coli communis is similar in length to the period of lag for other members of the colon-typhoid group in favorable media.

The bacteria present in the cultures of *Chilomastix* belong chiefly to the colon-typhoid group. Since some of these bacteria were used as food, the food supply of the flagellates was scarce during the lag phase of the growth of the bacteria. This scarcity of food supply might have been in part responsible for the period of lag in the growth of the flagellates when little or no multiplication occurred. The bacteria passing through the lag phase then entered upon a period of great multiplication. Within several hours the bacteria increased in number from a few hundred to many million. This great number of bacteria in the cultures furnished adequate food for the flagellates, and it seems only natural that the period of great fertility of the flagellates should now follow in the presence of this food supply.

Chemical decomposition products of bacterial metabolism have been forming in the cultures which have probably altered the culture medium in such a way as to make it most favorable for the growth of the protozoa. Because of these circumstances, it is very likely that the period of great fecundity of the flagellates was dependent to some extent upon the previous period of rapid multiplication of the bacteria, by virtue of which the culture medium was rendered favorable for the growth of the protozoa and an abundant food supply was obtained.

Table I shows that the rate of increase of the flagellates in the cultures began to decline after the 1st day. From the 3rd or 4th day on, the flagellates decreased in number and in most cases all had disappeared from the cultures on the 6th, 7th, or 8th day. This characteristic increase and decrease in the number of flagellates after the 1st day might also have been due to the metabolic relations existing between the protozoa and the bacteria, as appears to have been the case in the explanation of the great fecundity of the flagellates on the 1st day.

The chemical products of decomposition brought about by the metabolic activities of the bacteria accumulated rapidly during the first 24 hours, during which time the rate of multiplication of the bacteria was at its height. The amount of the chemical substances produced within this time was just sufficient to alter the medium

to the point where it was most favorable to the proliferation of the protozoa. Following the period of rapid reproduction of the bacteria, their rate of multiplication was slower and more or less constant; nevertheless, the chemical products were being formed and accumulated, but at a slower rate. Furthermore, mortality among the bacteria occurs at a slow rate in the early life of a culture but increases with the age of the culture. So in the culture of *Chilomastix*, as the death rate of the bacteria increased, the decomposition of their bodies produced other substances in the medium which might have been detrimental to the life of the flagellates.

Just as a minimum amount of these products of decomposition might have served to make the culture medium favorable for the rapid multiplication of the flagellates during the 1st day, so, through their accumulation from day to day, an excess of these substances may have become poisonous and thus inhibited the proliferation of the flagellates at a rapid rate after the 1st day. If this were the case, the continued accumulation of these now poisonous products caused a decrease in the number of the protozoa after the 3rd day and ultimately brought about the death and complete disappearance of the flagellates from the culture.

Some experiments have been performed upon amebæ, which, with the use of certain products of protein decomposition, resulted in an increased rate of cell division. The results of these experiments appear to lend evidence in support of the above theory that the chemical products of protein decomposition promoted the proliferation of the flagellates when present in small amounts, and that they became poisonous when present in larger amounts. The action of these substances as found in the experiments upon the ameba has well defined limits within which cell division is induced at a rapid rate, and beyond which death occurs.

The researches of Fantham[9] on *Entamœba coli*[10] showed that tyrosine, an aromatic compound and a product of protein putrefaction, when present to the

[9] Fantham, H. B., On the amœbæ parasitic in the human intestine, with remarks on the life-cycle of *Entamœba coli* in cultures, *Ann. Trop. Med. and Parasit.*, 1919, v, 111.

[10] Dobell (Dobell, C., The amœbæ living in man, London and New York, 1919) believed that the organism which Fantham cultured was not the parasitic ameba, *Entamœba coli*, but a free living species.

extent of 0.2 per cent in the culture medium, had the effect of shortening the life cycle, increasing the number of generations in any one culture, and increasing the frequency of binary fission.

Similarly the work of Cropper and Drew[11] on the cysts of the free living ameba, *Amœba ostrea*, showed that tyrosine (3 per cent) caused excystation in 24 hours; the free forms became larger, and among them were many multinucleate types. Choline, a member of the amine group and a product of the decomposition of amino-acids, showed even stronger powers in inducing cell division than the tyrosine. In one experiment two culture slides, A and B, contained equal volumes of 0.2 per cent choline in distilled water and distilled water, respectively. Upon A were 34 amebæ and on B, 26 amebæ. After 3 days the number of amebæ on A had increased from 34 to 207 and on B from 26 to 88. These investigators concluded that the substances "undoubtedly in certain strengths cause great proliferation of amœbæ," but also that when a certain strength of these substances is exceeded "the amœbæ are poisoned and multiplication is retarded."

In the study of the five cultures given in Table I, the reaction of the medium was changed from 0.1 per cent alkaline to phenolphthalein in the beginning, to 0.3 per cent alkaline at the end when all the flagellates had disappeared. The question arises as to whether or not the change in the reaction of the medium was responsible for the death of the flagellates. The change in the reaction may have been a contributing factor in the death of the flagellates, but at this time it does not seem fair to attach too much importance to this factor, because the flagellates thrived very well in some batches of medium with an initial reaction of 0.3 per cent alkaline to phenolphthalein which became more alkaline at the death of the culture. In view of this evidence it seems more probable that the death of the flagellates was brought about through the poisonous action of the chemical decomposition products of bacterial metabolism and not as a result of a change of the reaction of the culture medium.

In view of this theory, the persistent character of infections of man by *Chilomastix* may be explained in part. The accumulation of the products of protein decomposition does not normally occur in the intestine of man since the act of defecation carries them to the outside. The disappearance of the flagellates from the stools for short or long intervals might be explained by the death of numerous flagellates

[11] Cropper, J. W., and Drew, A. H., Researches into induced cell-reproduction in amœbæ, London, 1914.

through the toxicity of the substances of protein decomposition, temporarily accumulated, which in turn have altered the digestive tract to such an extent as to make it an unfavorable habitat. The removal of the substances would bring about a period of great proliferation which would be indicated by the presence of the flagellates in the stools again.

Reproduction in Cultures.

No detailed account of the methods of reproduction of *Chilomastix mesnili* will be given in this paper because of the inability thus far to study a sufficient number of stained preparations. It is hoped that at a later date this may prove possible. The observations to be recorded at this time were made chiefly upon living organisms, as the dividing forms were encountered during the examination of the cultures.

Binary Fission.—Binary fission was the type of cell division most often encountered, and in all probability it was the chief method of multiplication in the cultures after the 1st day. In the early stages of binary fission, the dividing flagellate was somewhat ovoid in shape, devoid of bacteria in most cases, and marked by the absence of a posterior protuberance. The first stage observed indicated an advanced period of division, for two nuclei were seen as bright refractile areas and a cytostome was present on either side. It could not be definitely determined whether a double set of anterior flagella was present or not, but there appeared to be a cytostomal flagellum for each oral pouch.

As the plasmotomy of the cytoplasm progressed, it was noticed that the plane of division was median and longitudinal. The separation of the two daughter flagellates began by a cleavage of the cytoplasm which proceeded anteroposteriorly. The division was apparently equal for both resulting organisms. The further stages in plasmotomy were aided considerably by the flagellar action of the organisms. The resulting daughter organisms tugged in opposite directions and the terrific lashing activity of the flagella no doubt served to separate the individuals. Just previous to their final separation, the two daughter flagellates were bound together by a fine, terminal strand of cytoplasm composed apparently of only the

hyaline periplast. The breaking of this strand set the organisms at liberty, while the resulting portions of the strand became the posterior spine for each flagellate. In one instance, approximately 35 minutes elapsed between the stage wherein plasmotomy had just begun to the final separation of the daughter flagellates.

From some stained preparations, there was noticed the formation of chromosomes, whose number appeared to be four, also the division of the centrosomes with the formation of a paradesmose. The paradesmose increased in length during the separation of the daughter nuclei. No study has been made of the different phases of mitosis, but the observations which have been made thus far have been in part recorded by Chalmers and Pekkola.[1]

Multiple Fission.—Evidence of multiple fission was also noticed in the cultures of *Chilomastix*. It is probable that this method of reproduction was the one most often resorted to during the 1st day, when the rate of multiplication was at its highest. The only flagellates apparently undergoing multiple fission were found during the first 22 hours in a culture which had been observed at intervals of an hour throughout 1 day and night until the following morning. Stained preparations were made, which revealed a few forms possessing four incomplete sets of organella.

In the living forms undergoing multiple fission, as in the case of binary fission, no posterior protuberance was visible. The body of the flagellate was large and spherical, in one instance measuring 19 microns in diameter. These forms did not appear to contain any bacteria, but the cytoplasm was more or less uniform in its granular constitution. The organism exhibited distinct ameboid movements by the bulging out and subsequent withdrawal of considerable areas of its body. The parts of the body which were so pushed out were usually equipped with flagella and a cytostome.

The body of the flagellate was a somatella comprising four zooids. The somatella possessed four distinct cytostomes located in different sectors of the body. Each cytostome had a cytostomal flagellum and nearby there appeared to be a single anterior flagellum. The nuclei were not seen in the living forms, but in the stained preparations the forms undergoing multiple fission possessed four nuclei. The nuclei were located in different sectors also, and near the cytostome of the respective sector.

Conjugation.—Whether or not other observations made upon *Chilomastix* in the cultures are to be interpreted as probable signs of conjugation can only be determined by further investigation. It seems advisable to record the observations of this nature made thus far.

It was noticed in the cultures, chiefly in those which were only 1 to 3 days old, that some of the flagellates were joined together in pairs. But while in this position, each flagellate was very actively beating with its flagella, and the manner of their union was such as even to permit the reversal of their respective positions. In the majority of these cases the common position of their bodies was such that their anterior ends were pointed toward the same direction, and when reversed the anterior end of each flagellate would point in the opposite direction. This reversal of direction occurred for only a moment, after which the original position was assumed again. Such forms were observed for a long time, but they were never seen to separate.

At present in the absence of more evidence from stained preparations one cannot attribute any syngamic significance to these double forms, since no actual transfer of nuclear substance was noticed. The two bodies of the flagellates in union did not apparently fuse together, for a line marking the confines of the cytoplasm of the organisms was always visible at the point of junction.

It must be stated, too, that clumping of the flagellates also took place at the edge of the cover-slip preparation, where the oxygen was more abundant. It does not seem, however, that the union of flagellates in pairs as described above was of the same nature as the clumping of the organisms in aggregates of a dozen and more in the area of the greatest abundance of oxygen. The paired forms were often seen after a sample had been taken directly from the cultures, at which time no clumping of the flagellates had occurred at the edge of the cover-slip.

SUMMARY.

1. Cases of flagellate infection of man by *Chilomastix mesnili* have been reported from nearly every locality in the world. They are fairly abundant in the United States and are often confused with cases of infection by *Trichomonas intestinalis*.

2. The shape of *Chilomastix* is pyriform. The body contains the following structures: nucleus, centrosome, primary, secondary, and tertiary blepharoplasts, parabasal, parastyle, peristomal fiber, ventral cytostome, cytostomal flagellum, three anterior flagella, food vacuoles with bacterial inclusions, and a posterior spine-like process, the holdfast organ.

3. The twisted shape in some flagellates in this case of infection is a temporary condition. It was seen in flagellates taken from the feces of the patient on only two occasions and was never observed among the cultured forms.

4. The cysts of *Chilomastix* are lemon-shaped and occurred irregularly in the stools of the patient. They have not been encountered thus far in the cultures.

5. *Chilomastix mesnili* was cultured continuously from January 27 to June 15, 1920, in an artificial medium composed of one part of human serum and four parts of Locke's solution with the addition of a small amount of dextrose.

6. Cultures sometimes remained viable for a period of 1 to 10 days, but generally for a period of 1 to 8 days.

7. The flagellates in the cultures increased in number during the 1st, 2nd, 3rd, and often the 4th days, after which there was a decrease until all had disappeared.

8. A period of lag, probably 13 hours in length, and followed by a period of great fecundity, characterized the growth of the flagellates during the 1st day in the cultures.

9. The number of flagellates in the cultures varied considerably and often reached 700,000 per cubic centimeter.

10. The bacteria served in part as the food of the flagellates. The bacterial organisms identified in the cultures were *Bacillus coli communis*, *Bacillus coli communior*, *Bacterium aerogenes*, *Bacillus alkaligenes*, and *Bacillus proteus*. The proteins of the blood serum, the mineral salts, and the dextrose in the medium may also have proved a source of food for the protozoa.

11. The products of bacterial metabolism, resulting from the growth of the bacteria, their fermentative activity, and their death may explain the rate of growth, reproduction, and death of the flagellates in the cultures. A certain amount of the products of decomposition

together with the presence of the bacterial food supply may explain the rapid proliferation of the flagellates during the 1st day and the slower rate up to the 3rd or 4th day. An excess of these products may have inhibited the growth and multiplication of the protozoa, especially after the 3rd or 4th day, and ultimately may have brought about their death and disappearance from the cultures.

12. The initial alkalinity of the medium increased at the death of the culture. It is not believed that the change in alkalinity proved lethal to the protozoa.

13. Binary fission was often observed. The plane of division was median and longitudinal. A paradesmose was formed.

14. Multiple fission also occurred. The flagellate body, ameboid in movement, was a somatella comprised of four zooids. It contained four cytostomes, each with a cytostomal flagellum. The number of anterior flagella was not complete for each zooid.

15. Flagellates, joined in pairs, were often seen during the first 3 days in the cultures. They may represent conjugating forms, but as yet no evidence of the exchange of nuclear substance has been seen.

MECHANISM OF UREA EXCRETION.

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PLATES 8 AND 9.

(Received for publication, August 2, 1920.)

Since the time of modern physiological investigation, two theories have dominated our conception of the mechanism of renal excretion. Bowman in 1842, basing his deductions on the anatomical structure of the renal unit, suggested that the glomerulus furnished the water of the urine, and that the solid substances were added to it by the activity of the renal cells which line the tubule. The experimental evidence for this view was furnished by Heidenhain[1] and his pupils in a long series of investigations.

2 years later, Ludwig[2] advanced his theory that all the urinary constituents were derived by filtration through the glomerulus, the ultimate concentration of the urine being arrived at by a process of absorption of water by the renal tubule. Both processes were held to be purely physical.

The various modifications which have been offered by the pupils of these two schools and by other observers are innumerable. The most recent concept is that of Cushny,[3] and is termed by him the modern theory. While neither of the original theories can explain the mass of physiological and morphological data that has accumulated since their origin, the modern theory covers in a much more satisfactory manner our present knowledge.

According to Cushny, the formation of the urine may be explained by two processes: first, a purely physical filtration through the glomerulus of all the constituents of the plasma except the colloids; and, second, a resorption from this filtrate by the vital activity of the epithelium as it passes down the tubule. The former process furnishes the urinary constituents, the latter modifies their amounts so that they correspond to those of the completed urine.

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[1] Heidenhain, R., in Hermann, L., Handbuch der Physiologie, Leipsic, 1883, v, 279.

[2] Ludwig, C., Lehrbuch der Physiologie des Menschen, Leipsic and Heidelberg, 1861, ii, 373.

[3] Cushny, A. R., The secretion of the urine, London and New York, 1917.

Water is obviously the substance which will be handled in greatest amount by both filtration and absorption, and the conception of such a double process with this substance furnishes little difficulty. A complication arises, however, when the solid constituents are considered, as a uniform rate of absorption will not explain the varying levels of concentration that the different solids show in the completed urine. These are therefore divided into "threshold" and "non-threshold" substances. The former appear in the urine only as they exceed a certain value below which they are completely absorbed. Sugar and the chlorides are examples. The non-threshold substances, on the other hand, are found in the urine in direct proportion to their absolute amounts in the plasma, as they are not absorbed. Of these, urea and sulfates are the most important. The urine therefore contains all the urea of the glomerular filtrate and none of the sugar.

TABLE I.

Amount of Filtrate and Absorbed Fluid Required to Form 1 Liter of Urine.
Blood urea 70 mg. per 100 cc.; urine urea 5.5 per cent; urine 1,000 cc.

	Urea.	Water.
	gm.	*cc.*
Blood	0.07	100
Glomerular filtrate	55.0	78,500
Amount absorbed by tubule	0.0	77,500
Urine	55.0	1,000

Table I shows the amount of filtrate and absorbed fluid which would be required to form 1 liter of urine in an actually observed case of high urea excretion. The figures in the table hold only in case the entire urea content of the blood is available for filtration. In a recent article Cushny[4] claims that as the urea is distributed in about equal amount between the plasma and the corpuscles, only that half free in the plasma can pass through the glomerular filter. In this case, to obtain the 55 gm. found in the urine, twice as much filtrate would be needed as is shown in the table; 157,000 cc. of filtrate would be formed, of which 156,000 cc. would be absorbed.

Such a mechanism, though perhaps indirect, will nevertheless account for those alterations which would be required to convert the

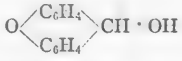
[4] Cushny, A. R., *J. Physiol.*, 1919–20, liii, 391.

blood plasma to urine without the intervention of a secretory factor. The completeness with which the various experimental and clinical facts are covered by this theory is well shown by Cushny.[3] Even changes in the mitochondria and the appearance of dyes in the tubule cells, which Heidenhain considers morphological evidence of a secretory process, may be equally well accounted for by the assumption of a vital absorption from the lumen of the tubule. This also applies to the demonstration of uric acid in the tubule cells, as this substance is one of the threshold group, and, according to the modern theory, is absorbed from the lumen of the tubule during concentration of the urine.

Urea, however, is a non-threshold substance and, if demonstrated in the renal cells, cannot be explained by a process of absorption from the lumen. The concentration of urea in the glomerular filtrate must be raised to that of the urine, and this could be done slowly, or not at all, if urea was absorbed along with the water.

Urea has been demonstrated in the proximal convoluted tubule by Leschke[5] and his experiments have been confirmed by the writer.[6] The method, which depends on the formation of an insoluble compound between urea and mercuric nitrate, may be criticized, as the resulting reaction is not so distinctive as might be desired. The protoplasm of all cells reacts more or less, though always less than that of cells of the proximal convoluted tubules. Whether this is due to the delicacy of the reaction, for Marshall and Davis[7] have shown the widespread distribution of urea in all tissues, or to a lack of specificity, is impossible to say. The quantitative variation in the degree of the reaction in the cells of the proximal convoluted tubule depending on the concentration of urea in the secreted urine has been taken as evidence of its specificity.[6]

Subsequently a new qualitative reagent for urea has been described by Fosse.[8] This is xanthydrol



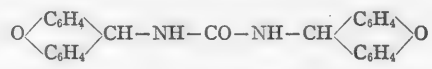
[5] Leschke, E., *Z. klin. Med.*, 1915, lxxxi, 14.

[6] Oliver, J., *J. Exp. Med.*, 1916, xxiii, 301.

[7] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

[8] Fosse, R., *Bull. sc. pharmacol.*, 1914, xxi, 74, 502.

which forms with urea a characteristic crystalline product



The crystals are insoluble in acetic acid and differ in this way from the compounds which xanthydrol forms with other substances.

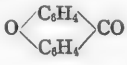
Two attempts have been made to demonstrate urea in the tissues with this reagent. Policard[9] was unable to find the crystals in any of the injected kidneys, though they were present in the lumina of the collecting tubules. He therefore concluded that urea does not exist in a free form in the kidney cells, but that it is bound in some intimate combination with the protoplasm. Chevallier and Chabanier,[10] however, describe the typical crystals of the urea compound in the cells of the convoluted tubules, in all the vessels of the kidney, and in the lumina of the ducts of Bellini.

The importance of these findings with regard to the mechanism of urea excretion, as well as the need for the accurate localization of the urea in the various parts of the kidney, is obvious. For these reasons the experiments have been repeated with certain modifications.

Technique.

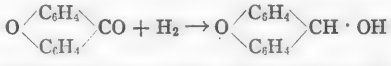
Reagents.—As it is impossible to obtain xanthydrol in the market, it was prepared in the following manner.

100 gm. of salol are heated to boiling in a distillation flask and the first fluid fraction, consisting largely of phenol, of 35 to 40 gm., is discarded. The remaining fraction, xanthone,



comes over and condenses in the form of long needles. These are collected and heated with NaOH, washed free of alkali, and purified by recrystallization from alcohol.[11] The xanthone gives a bright yellow color with a light blue fluorescence if treated with concentrated H_2SO_4.

The next step consists in the reduction of xanthone to xanthydrol.



[9] Policard, A., *Compt. rend. Soc. biol.*, 1915, lxxviii, 32.

[10] Chevallier, P., and Chabanier, H., *Compt. rend. Soc. biol.*, 1915, lxxviii, 689.

[11] Vanino, L., Handbuch der präparativen Chemie, Stuttgart, 1914, ii, 512.

This is accomplished by boiling in a reflux condenser 10 gm. of xanthone, 40 gm. of NaOH, and 400 cc. of alcohol, adding from time to time a pinch of zinc dust so that there is always a small amount present. The process is continued for from 6 to 8 hours, and the fluid then poured into cold water. The xanthydrol comes down in the form of fine crystals which are washed and dissolved in boiling alcohol and recrystallized by pouring again into an excess of water.[12] The xanthydrol gives with concentrated H_2SO_4 a bright yellow color with a light green fluorescence.

The mixture used for the injection of the kidneys was made up fresh for each experiment, as follows: 2 gm. of xanthydrol were triturated with 10 to 15 cc. of methyl alcohol, and 20 cc. of glacial acetic acid were added. The turbid fluid was filtered and was then a clear light yellow. If added to water, the xanthydrol comes out of solution, and the same precipitation occurs to some degree in the tissues. These crystals, however, are soluble in alcohol, and their disappearance may be easily followed as the specimen passes through the alcohols during embedding and staining.

Animals Used.—Rats were used for the experiments as their kidneys require less reagent than those of larger animals. In order to get a satisfactory concentration of urea in the urine, some animals were either fed a mixture of lard and corn meal containing urea, or a dilute solution of urea was injected into the peritoneal cavity. Others were injected after having lived on a normal diet. The first procedure was preferred as more nearly approaching physiological conditions. The animal was killed, the thorax quickly opened, and the reagent injected by way of the aorta. The injection was continued until the kidney was completely fixed. Thin slices were made and placed over night in 95 per cent alcohol, the tissue was embedded in paraffin, and sections were stained with hematoxylin.

EXPERIMENTAL.

If a rat whose kidney is excreting urine high in urea concentration is injected with the xanthydrol reagent, the organ swells somewhat and turns a light opaque yellow. On the cut surface one can see

[12] Meyer, R., and Saul, E., *Ber. chem. Ges.*, 1893, xxvi, 1276.

with a hand lens that this discoloration is due to the presence of innumerable minute crystals scattered throughout the cortex and appearing in the medulla in long thin stripes.

Microscopic examination of the sections shows the exact location of these crystals. They lie in three places: in the blood vessels, in the cells of certain tubules, and in the lumina of the tubules (Figs. 1 to 5).

The Intravascular Crystals.

In all parts of the kidney and in the surrounding tissues (perirenal fat), all blood vessels contain typical crystals (Figs. 1, 3, and 5). These are in the form of fine, pointed needles, varying in size from 4 to 50 μ in length, lying either singly or arranged in the form of rosettes. On account of the arrangement of the vessels, the crystals appear in the medulla in long rows between the straight tubules, while in the tortuous capillaries of the cortex they are more irregularly scattered. They are also seen in the capillaries of the glomerular loop (Fig. 1).

The number of crystals in the individual cross-sections varies greatly; some are empty, others completely filled. This irregular distribution is evidently due to the current of the injection fluid which carries the crystals with it.

The Intracellular Crystals.

Crystals contained in cells are found only in the cortex, where the thick epithelium of the proximal convoluted tubule is filled with them (Fig. 1). They lie in all parts of the cell, near the membrana propria, in the region of the nucleus, or just beneath the brush border (Fig. 2). They are smaller than those seen in the blood, and when arranged in rosettes are more or less deformed. Their characteristic structure is nevertheless evident.

The number of crystals in the tubule cells gradually decreases so that the terminal spiral portions of the proximal tubule show definitely fewer crystals than the cross-sections which lie in the proximity of the glomerulus.

The other divisions of the renal tubule practically never show intracellular crystals. This contrast is not so evident in the cortex, where the great majority of the cross-sections are of the proximal convoluted

tubule, but at the junction of the outer and inner stripes of the outer zone of the medulla it is striking. Here the large spiral terminal portions of the proximal convoluted tubules, situated in the outer stripe, end suddenly to form the line of demarcation from the inner stripe, which contains the broad ascending limbs of Henle's loop. The former contain the crystals in moderate number, while the latter do not show them (Fig. 3).

The Crystals in the Lumen of the Tubule.

The lumen of the entire renal unit, from the capsular space to the large ducts of Bellini, contain the crystals, which resemble in their long needle shape those seen in the blood. The increase in number and in the size of the rosettes toward the end of the tubule is striking.

In Bowman's space the crystals are comparatively small and infrequent (Fig. 1). Beginning in the lumen of the proximal convoluted tubule, however, there is a definite increase in their number, which becomes even greater in the narrow descending limb of Henle's loop (Fig. 4). The sections of the small collecting tubules in the cortex show an added increase (Figs. 1 and 3), while in the terminal ducts of Bellini relatively large rosettes are seen (Fig. 5).

As in the case of the blood vessels, not every cross-section of a tubule shows an equal amount of crystals. In those regions where the urine is comparatively dilute, as in the proximal convoluted tubules, many are empty. This may be explained by the fact that a volume of urine produces a relatively much smaller volume of crystalline product, so that as the urea is condensed in crystalline form, it leaves the surounding areas free. A single rosette of crystals may thus represent the urea from a considerable length of tubule.

Demonstration of Urea Crystals in Other Tissues.

As a control, the livers of certain of the animals were injected with the reagent. Here the hepatic vessels contained the crystals in the same amount as those of the kidney; the hepatic cells, however, did not show them.

DISCUSSION.

The interpretation of the above findings is greatly aided by our accurate knowledge of the distribution of urea in the body. It has been shown that all the tissues except the fat contain urea in the same concentration as that found in the blood, the kidney alone exceeding this amount (Marshall and Davis[7]).

From this it follows that the concentration of urea in the cells of the proximal convoluted tubules, which show the crystals of urea, must be higher than that of the blood, as in the liver and the other tubules of the kidney, where the concentration is equal to that of the blood, there is no reaction.

There is therefore a certain threshold below which the reaction does not take place in the protoplasm. The deformity and small size of the intracellular crystals is further evidence of this embarrassment to the reaction. This threshold lies somewhere above the concentration of urea in the blood, and is only reached in the proximal convoluted tubule.

The question now arises as to whether the source of this excess urea is the lumen of the tubule (absorption) or the blood (excretion).

Any theory concerning the mechanism of urea excretion should explain the great rise in urea concentration of the urine as contrasted with that of the blood. By a process of absorption this can only be accomplished if the other constitutents of the urine (water) are absorbed and the urea is left behind, and such an assumption is made by the modern theory. Urea would not, therefore, be found in a higher concentration in the cells of the tubule than in the blood.

The excretion of urea, on the other hand, will raise the concentration in the urine, and the high concentration in the cells would be expected. Our demonstration of such a high concentration can therefore only be explained by the assumption of an excretion of urea from the blood into the lumen of the tubules.

That absorption of water without absorption of urea takes place, however, is shown by the increased concentration of urea in the urine as the tubule is descended so that large rosettes of crystals are seen in the ducts of Bellini but none in the epithelium (Fig. 5).

The mechanism of urea excretion may be summarized as follows: Urea passes through the glomerular capsule in the same concentration as that found in the plasma. A certain amount is added to this filtrate by excretion through the cells of the proximal convoluted tubule, and the ultimate concentration is reached by an absorption of water in the remainder of the tubule.

Such a modification will not affect the fundaments of the modern theory, as Cushny states that "it may be necessary to supplement what I have termed the modern view with an active secretion in the tubules."[13]

CONCLUSIONS.

1. Urea is present in the cells of the proximal convoluted tubule in a concentration higher than that of the blood or than that of the cells of any of the other kidney tubules.

2. Such a condition can only be reconciled to an assumption of an active secretion (excretion) on the part of these cells.

3. Urea also passes through the glomerular filter with the other crystalloids of the blood plasma.

4. The final concentration of urea is due to the above mentioned secretion by the proximal convoluted tubule, and to the absorption of water in other parts of the tubule.

EXPLANATION OF PLATES.

Drawings made with the aid of a camera lucida. Bausch and Lomb ocular 1, objective $\frac{1}{6}$.

PLATE 8.

FIG. 1. Rat 1. 2 cc. of 5 per cent urea intraperitoneally; killed 1 hour later. A glomerulus is shown with several sections of the proximal convoluted tubule and one of a collecting tubule. Crystals of urea-xanthydrol are seen in the vessels near the glomerulus, in the loops of the capillary tuft, in the space of Bowman's capsule, and in greater number in the cells of the proximal convoluted tubule. The lumen of the collecting tubule also contains some rosettes of crystals.

FIG. 2. Rat 1. Higher magnification of one of the proximal convoluted tubules. Small rosettes of crystals are seen scattered throughout the epithelial cells. There are some crystals in the vessels. Bausch and Lomb ocular 1, objective $\frac{1}{12}$.

[13] Cushny,[3] p. 52.

FIG. 3. Rat 2. Same as Rat 1. The junction of the inner and outer stripes of the outer zone of the medulla is shown. Above, the larger terminal ends of the proximal convoluted tubules containing crystals in their cells, and in one case a rosette of crystals in the lumen. Below, the smaller ascending limbs of Henle's loop which contain no crystals. Scattered crystals and rosettes are seen in the intertubular capillaries and in the collecting tubule at the left.

PLATE 9.

FIG. 4. Rat 3. Fed a mixture of urea (10 per cent), corn meal, and lard for 3 days; killed and injected. The tubules represented were situated in the inner stripe of the outer zone of the medulla. Four ascending limbs of Henle's loop are shown surrounding the loop proper which is formed by the narrow limb of the tubule in this case. Its lumen contains many crystals.

The loop shown in this figure is one of the short type described by Peter,[14] in which the bend lies close to the end of the proximal convoluted tubule. There could have been, therefore, little opportunity for the absorption of water to produce the relatively high concentration of urea as indicated by the mass of crystals.

FIG. 5. Rat 4. Rat on normal diet, no urea given. Margin of the papilla of the medulla. Three large ducts of Bellini are shown, their lumina filled with huge rosettes of crystals. Scattered rosettes are also seen in the pelvis, and a few in the intertubular capillaries.

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[14] Peter, K., Untersuchungen über Bau und Entwickelung der Niere, Jena, 1909.

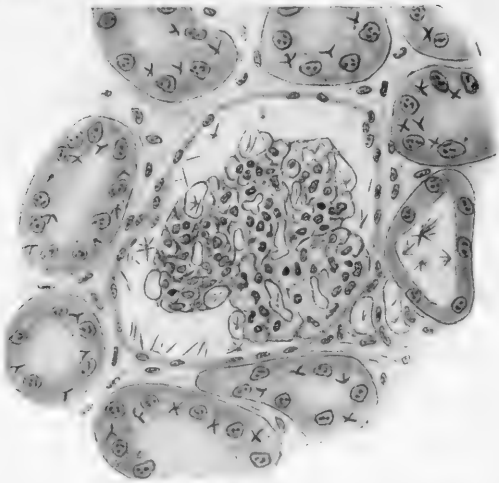

FIG. 1.

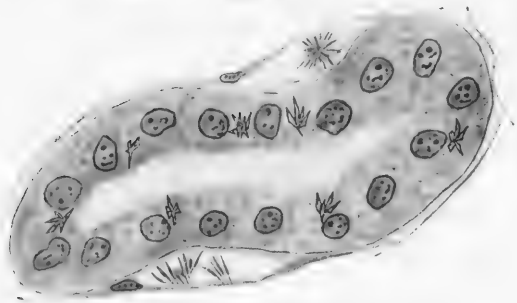

FIG. 2.

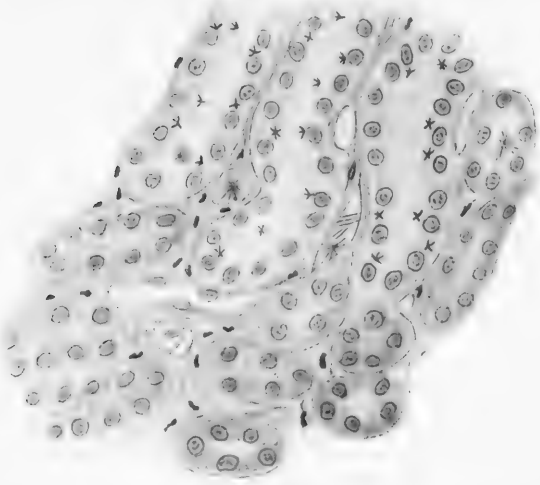

FIG. 3.

(Oliver: Mechanism of urea excretion.)

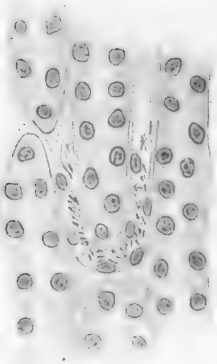

FIG. 4.

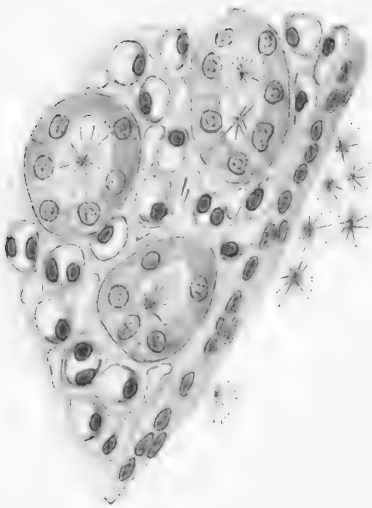

FIG. 5.

IMMUNOLOGICAL DISTINCTIONS OF ENCEPHALITIS AND POLIOMYELITIS.

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(Received for publication, August 26, 1920.)

In the course of the many discussions of encephalitis lethargica which have followed the pandemic of that unusual disease, the question of its relation to epidemic poliomyelitis has been raised either incidentally or directly. Von Economo,[1] who reported the first Austrian cases, believed that poliomyelitis could be excluded, while Draper,[2] who studied the residual pareses in some of the cases in the English outbreak of 1918, concluded that many of them were true cases of poliomyelitis; however, he called attention to certain phenomena distinctly unusual in experiences with poliomyelitis and left open the question as to whether in the whole group there existed a subsection representing a new disease. Crookshank,[3] in discussing the epidemiology, has expressed the view that poliomyelitis, lethargic encephalitis, and possibly still other paralytic epidemic affections, may be different manifestations of one etiologically simple malady.

There are grave objections to the confusion of the etiology of poliomyelitis and lethargic encephalitis. The divergent clinical manifestations have been more marked as the epidemic outbreak of lethargic encephalitis has endured and become more widely distributed. The great difference in the communicability of this affection as compared with poliomyelitis is another point of capital distinction. Apparently the seasonal variation in the prevalence, namely the occurrence of poliomyelitis in the summer and autumn months and

[1] von Economo, C., *Wien. klin. Woch.*, 1917, xxx, 581.

[2] Draper, G., *Rep. Local Gov. Bd. Pub. Health and Med. Subjects, New Series, No. 121*, 1918, 62.

[3] Crookshank, F. G., *Boston Med. and Surg. J.*, 1920, clxxxii, 34.

of lethargic encephalitis in the winter and spring months is no longer so sharp. Netter[4] has reported summer cases of the latter malady in Paris. Moreover, from information furnished by the Department of Health of the City of New York and the statistics in the *Public Health Reports of the United States Public Health Service* it appears that a similar extension of the period of prevalence is occurring in the United States.

There is one means of distinguishing epidemic poliomyelitis and lethargic encephalitis which has not yet been applied. It relates to the point whether the serum of convalescent cases of lethargic encephalitis can neutralize the virus of poliomyelitis. This fact is readily determined experimentally by the method described by Amoss and Eberson.[5] The principle of the test lies in the power of a neutralizing serum, when administered intraspinally, to prevent the development of poliomyelitis in the monkey following the intravenous injection of a large dose of the virus.

EXPERIMENTAL.

The blood serum of four cases of lethargic encephalitis was used in the test, one from a patient convalescent in the 5th week of the disease, the second in the 4th month, the third in the 5th month, and the fourth 15 months after the attack. The tests were controlled by two experiments in which the same procedure was followed, except that one monkey received intraspinal injections of normal human serum and the other intraspinal injections of poliomyelitic serum from a monkey which had had experimental poliomyelitis 9 months before and recovered with residual paralyses.

The virus of poliomyelitis used came from a strain which had been passed from monkey to monkey many times during the past 9 years and which, between passages, had been preserved in 50 per cent glycerol in the ice box. Before starting the tests this virus was passed through three normal monkeys in order to determine its virulence. The certain infecting dose for intracerebral injection[6]

[4] Netter, A., *Bull. et mem. Soc. méd. hôp. Paris*, 1920, xliv, series 3, 1030.

[5] Amoss, H. L., and Eberson, F., *J. Exp. Med.*, 1918, xxvii, 309.

[6] All intracerebral inoculations were made under ether anesthesia.

was found to be 0.25 cc. of a Berkefeld filtrate of a 5 per cent suspension of the nervous tissues containing the virus.

The infecting power of the virus when given intravenously is shown in the following preliminary experiment.

A monkey received at 5 p.m. an intraspinal injection of 2 cc. of normal horse serum. The following morning 50 cc. of a 5 per cent suspension of fresh virus were given intravenously. 5 days later the animal was weak in both legs and excited. Both arms were paralyzed on the 6th day and the monkey died on the 7th day.

Autopsy.—Microscopic lesions of experimental poliomyelitis were found.

In making suspensions for intravenous injections the tissues used must be fresh. Accordingly, a monkey prostrate from an intracerebral injection of 0.5 cc. of a suspension of the virus 6 days before, was killed with ether and autopsied at once. A 5 per cent suspension of the cord and medulla was prepared for immediate injection.

Series 1.

Experiment 1.—Macacus rhesus A. Normal human serum control. Mar. 11, 1920, 5.50 p.m. Injected intraspinally 2 cc. of fresh normal human serum. Mar. 12, 2.30 p.m. Injected intravenously 50 cc. of a 5 per cent suspension of fresh poliomyelitis nervous tissue. 2.50 p.m. Intraspinal injection of 2 cc. of fresh normal human serum. The intraspinal injection of 2 cc. of normal human serum was repeated daily for 3 days. Mar. 18. Excited; slight head tremor and left facial paralysis. Mar. 19. Prostrate. Mar. 20. Etherized when moribund.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 2.—Macacus rhesus B. Immune poliomyelitic serum. Mar. 11, 1920, 6 p.m. Injected intraspinally 2 cc. of serum from a monkey which had had experimental poliomyelitis 9 months before and had recovered with residual paralyses. Mar. 12, 3 p.m. Injected intravenously 50 cc. of virus suspension. 3.25 p.m. Intraspinal injection of 2 cc. of poliomyelitic immune monkey serum. The intraspinal injections of 2 cc. of immune monkey serum were repeated daily for 3 days. The monkey remained well.

Experiment 3.—Macacus rhesus C. Serum from convalescent case of lethargic encephalitis. Mar. 11, 1920, 6.05 p.m. Injected intraspinally 2 cc. of serum from Case 1, age 31 years, who was in the 5th week of well defined lethargic encephalitis with general disturbance of the functions of the central nervous system and involvement of third and seventh cranial nerves. Mar. 12, 3.30 p.m. Intravenous injection of 50 cc. of virus suspension. 3.55 p.m. Intraspinal injection

of 2 cc. of encephalitis serum from Case 1. The intraspinal injection of the encephalitic serum was repeated daily for 3 days. Mar. 17. Animal slow and weak. Mar. 18. Found dead at 9 a.m.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 4.—Macacus rhesus D. Serum from convalescent case of lethargic encephalitis. Mar. 11, 1920, 6.10 p.m. Injected intraspinally 2 cc. of serum from Case 2, age 34 years, 3 months after definite attack of lethargic encephalitis in which there was general disturbance of the function of the central nervous system, involving the third and seventh cranial nerves and spinal motor roots. Mar. 12, 4 p.m. Intravenous injection of 50 cc. of virus suspension. 5 p.m. Injected intraspinally 2 cc. of convalescent encephalitis serum from Case 2. The intraspinal injection of 2 cc. of encephalitic serum was repeated daily for 3 days. Mar. 18. Excited and ataxic. Mar. 19. Prostrate. Mar. 20. Etherized when moribund.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Series 2.

The second series of tests carried out at a different time was controlled by an experiment in which normal human serum was used for the intraspinal injections as in Experiment 1. The procedure in this series was the same as in Series 1, except that normal horse serum was used for the preparatory intraspinal injection given the day before the intravenous injection of virus. The same strain of virus employed in Series 1 was again tested for infecting power and used in this series.

Experiment 5.—Macacus rhesus E. Normal human serum control. May 18, 1920, 4.10 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 12 m. Intravenous injection of 50 cc. of virus suspension. 12.25 p.m. Injected intraspinally 2 cc. of normal human serum. The intraspinal injection of normal human serum was repeated daily for 3 days. May 23. Monkey excited; both legs weak. May 24. Complete paralysis of both legs and right facial paralysis. May 25. Died.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 6.—Macacus rhesus F. Serum from case of lethargic encephalitis. May 18, 1920, 4 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 11.30 a.m. Intravenous injection of 50 cc. of virus suspension. 11.55 a.m. Intraspinal injection of 2 cc. of serum from Case 3, age 28 years, taken 4½ months after acute onset of lethargic encephalitis. The patient's illness began with dizziness, disturbance of vision, vomiting and fever, and he gradually became stuporous. Later paralyses referable to the seventh and spinal nerves appeared. The intraspinal injection into the monkey of the convalescent serum was repeated

daily for 3 days. May 26. Monkey had paralysis of both arms and shoulder muscles. May 27. Died.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 7.—Macacus rhesus G. Serum from case of lethargic encephalitis. May 18, 1920, 4.20 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 11 a.m. Intravenous injection of 50 cc. of virus suspension. 11.25 a.m. Injected intraspinally 2 cc. of serum from Case 4, age 24 years, who had developed lethargic encephalitis 15 months before and was still under observation with residual ptosis and partial paralysis of left side of face and left leg. The intraspinal injection into the monkey of the convalescent encephalitis serum was repeated daily for 3 days. May 24. Monkey had double ptosis. May 25. Shoulder muscles paralyzed; arms and legs very weak; head tremor; animal almost prostrate. May 26. Died.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

CONCLUSIONS.

Lethargic encephalitis is an epidemic disease, the main manifestations of which relate to injury inflicted upon the central nervous system and in particular the basal ganglia of the brain.

Poliomyelitis is an epidemic disease, the main manifestations of which relate to injury inflicted upon the central nervous system and in particular the gray matter of the spinal cord and medulla oblongata.

At the outset of the epidemic of lethargic encephalitis the two diseases tend to prevail at distinct and different seasons of the year, although recently cases of epidemic encephalitis have arisen in the midsummer months. The two maladies therefore are perhaps less distinguished by seasonal prevalence than has been supposed.

They are, however, distinguished by great differences in communicability to monkeys. Epidemic poliomyelitis is readily transmitted through inoculation of the affected central nervous tissues of man to monkeys, while it may still be regarded as doubtful whether lethargic encephalitis has been communicated to monkeys in this manner.

As the experiments reported in this paper show, the two diseases can be distinguished through the power of blood serum under certain circumstances to neutralize the virus of poliomyelitis. The blood serum of convalescent cases of poliomyelitis whether in man or monkey

possesses this neutralizing power, while the blood serum of recently convalescent cases of epidemic encephalitis is devoid of it.

On the basis of the distinguishing characters described, it is regarded as desirable at the present time to hold epidemic poliomyelitis and epidemic encephalitis as integrally distinct affections. The latter also may be infectious, yet the main lesions of poliomyelitis are present in the spinal cord, and of epidemic encephalitis in the mid-brain.

THERAPEUTIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID (TRYPARSAMIDE) UPON EXPERIMENTAL INFECTIONS OF TRYPANOSOMA RHODESIENSE.

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(Received for publication, October 13, 1920.)

In a series of papers published in November, 1919 (1), we reported results which had been obtained from the treatment of various forms of experimental trypanosomiasis with *N*-phenylglycineamide-*p*-arsonic acid, or tryparsamide. The results were such as to indicate that this drug might prove of value in the treatment of the form of human trypanosomiasis which is due to infection with *Tr. gambiense*. At the time this work was done, *Tr. rhodesiense* was not obtainable for experimental study, but as the therapy of the infections caused by this organism is becoming of increasing importance, it was deemed advisable to determine the effects which might be hoped for from the use of this drug in the treatment of Rhodesian sleeping sickness, and something of the method of treatment which might be employed in these cases.

Accordingly, a strain of *Tr. rhodesiense* was obtained and a series of experiments carried out upon various forms of the animal infection including that in mice, rats, and rabbits.

Tr. rhodesiense usually exhibits a higher degree of virulence for laboratory animals than *Tr. gambiense*. The organism used in these experiments, however, was distinctly less virulent for all the animals used, except the rabbit, than the strains of *gambiense* with which we had worked. After serial passage through mice and rats, it developed a fair degree of virulence for both species of animals and was highly virulent for rabbits, but possessed very slight virulence for guinea pigs. When inoculated into mice and rats according to the procedure described in a previous paper (2), the incubation period of the infection

was from 2 to 3 days in the case of mice and 3 to 5 days in rats. Infected mice died in from 10 to 12 days, while rats survived a few days longer. Rabbits were also readily infected; well marked symptomatic manifestations of disease developed in less than a week after intravenous inoculation, and the infection terminated fatally within 2 to 4 weeks.

Attempts to transmit the infection to guinea pigs were at first unsuccessful, but eventually a low grade infection with a prolonged incubation period and a low mortality was produced and serial passages were maintained through several generations of transfers.

EXPERIMENTAL.

The general plan of the therapeutic experiments carried out was the same as that employed in our previous work; that is, the trypanocidal action of the drug was studied by the use of the simpler blood stream infections of mice and rats, and the results thus obtained were applied to the treatment of the more complex disease as it appears in the rabbit. Mice and rats were inoculated intraperitoneally and treated 24 hours later by the intraperitoneal administration of a single dose of the drug. Repeated blood examinations were made to determine the effects upon the infecting organisms. Mice were kept under observation for 1 month after treatment and rats for 2 months, as a means of determining the probable results of treatment.

Rabbits were inoculated intravenously and treated by the same route when well marked symptoms of disease had developed; the period of observation was 3 months, although most of the animals in this series were held as long as 4 months.

Therapeutic Effects.

Mice.—Several series of mice were treated with doses of from 0.15 to 1.75 gm. per kilo of body weight. Doses below 0.25 gm. per kilo were found to exercise very little if any effect upon the general course of the infection and those of from 0.25 to 0.5 gm. merely served to delay its progress. No cures were obtained until the dose of drug given reached 0.75 to 1 gm. per kilo. As an example of the therapeutic effects obtained, the detailed results of one of the experiments are given in Table I.

TABLE I.

*Results from the Treatment of a 24 Hour Infection of Tr. rhodesiense in Mice.**

Dose per kilo.	No. of mice.	No. of relapses.	No. of probable cures.
gm.			
1.75	2	0	2
1.50	2	0	2
1.25	2	1	1
1.00	2	2	0
0.75	2	1	1

* Controls survived 11 days.

Rats.—It was found that the rat infection of *Tr. rhodesiense* was also difficult to influence with this drug. The results obtained from experiments carried out under the same conditions as with mice may be illustrated by the experiment given in Table II.

TABLE II.

Results from the Treatment of a 24 Hour Infection of Tr. rhodesiense in Rats.

Dose per kilo.	No. of rats.	No. of relapses.	No. of probable cures.
gm.			
0.75	3	0	3
0.60	3	2	1
0.50	3	1	2
0.35	3	3	0

While smaller unit doses of the drug were capable of curing the infection produced in rats than in mice, when considered from the standpoint of the ratio of the curative to the lethal dose, the figures obtained in the two instances were in very close agreement, with a slightly better ratio for the mouse. Thus it was found necessary to administer upwards of two-thirds of the maximum dose in order to effect a complete sterilization in the case of the mouse, and while cures might be obtained with only two-thirds of the maximum dose in rats, the full dose was necessary to assure such a result.

These figures were quite different from those which had been obtained with *Tr. gambiense* under similar conditions. Comparable results were obtained in mouse and rat infections of this organism

in doses as small as one-ninth and one-fifth respectively of the maximum dose for the two animal species. It appeared, therefore, that greater difficulty might be anticipated in the treatment of the more severe chronic infection of rabbits.

Rabbits.—In tests of the therapeutic effects against *rhodesiense* infection in rabbits, the animals used were inoculated intravenously with 1 cc. per kilo of body weight of a + to + + suspension of trypanosomes prepared from rat blood. This produced a very intense infection, symptoms appearing in individual animals within 3 to 5 days after inoculation, and the majority of the animals showed well marked manifestations of disease by the end of the 1st week. The controls of the series survived for only 2 weeks. Three types of experiments

TABLE III.

Results from the Treatment of Tr. rhodesiense Infections in Rabbits by the Intravenous Administration of a Single Dose of Tryparsamide Given 1 Week after Inoculation.

Dose per kilo.	No. of rabbits.	No. of relapses.	No. of probable cures.
gm.			
0.75	3	1*	2
0.60	3	2†	1

* Relapse 19 days after treatment.

† Relapse 19 and 22 days after treatment.

were undertaken in this series: (1) the use of large single doses, (2) the use of repeated smaller doses, and (3) an intensive treatment of relapses.

Single Doses.—In view of the marked resistance exhibited by *Tr. rhodesiense* in mice and rats, the single dose treatment of rabbits was placed at the highest possible level consistent with safety. A small series of rabbits was treated, with the results recorded in Table III.

Repeated Doses.—The attempt was also made to determine whether any form of repeated dose therapy based upon the use of smaller doses than those described above might prove efficacious in this class of infection. Two rabbits were given doses of 0.4 gm. per kilo at intervals of 3 days. One of these received six doses, and after a rest

period of 1 week, the treatment was resumed with nine doses at intervals of 2 to 3 days. The animal thus received a total dose of 6.0 gm. of the drug per kilo of body weight within a period of 40 days. With the second animal the number of doses was reduced to six, or a total amount of 2.4 gm. of the drug per kilo was given within 15 days. Both these animals showed rapid improvement and remained in excellent condition throughout the course of treatment. Signs of the disease disappeared completely and there was no recurrence within the period of observation. They may, therefore, be regarded as probable cures.

The same procedure was followed with three other rabbits with a still smaller unit dose of the drug—0.3 gm. per kilo. One animal received six doses of the drug at intervals of 3 days, or a total of 1.8 gm. in 15 days; the second received five doses followed by a rest period of 1 week and then a second series of ten doses, or a total of 4.5 gm. was given in 43 days; the third rabbit was given fifteen consecutive doses at regular intervals, the 1 week interim between series being omitted.

The first of these animals did well under the treatment; symptoms of disease disappeared promptly and there was no recurrence. The initial effects were much the same in the second animal, but although the course of treatment was much more prolonged, it proved ineffectual. Within 4 days after administration of the drug had been stopped, signs of the disease reappeared, and the animal died from trypanosomiasis 15 days later. With the third rabbit, which was given the same amount of drug in consecutive doses, a cure was effected.

This small group of cases furnishes an excellent illustration of the variability of individual results which may be obtained in the treatment of infections of this type and possibly also the effects which may be produced by apparently minor variations in the course of treatment.

Relapses.—In view of the great difficulty experienced in influencing *rhodesiense* infections, little was to be expected from an attempt to treat these infections once they had relapsed. It was considered worth while determining, however, whether it was possible to obtain any effect from the use of the drug in this class of cases. Three experiments of this kind were carried out.

The first case treated was a rabbit which had relapsed from a single dose of 0.75 gm. per kilo. On the 3rd day after the symptoms of the disease reappeared, treatment was begun with 0.5 gm. per kilo, and nine doses of the drug were administered at intervals of 2 to 3 days, or 4.5 gm. per kilo in 21 days. This was considered to be as intensive treatment as could be undertaken and in this instance apparently effected a cure. The animal when inoculated weighed 1,625 gm.; when retreated after relapse, 1,600 gm.; when treatment was discontinued, 1,875 gm.; when discarded, 1,900 gm. This experiment furnishes an excellent example of the remarkable tolerance exhibited by animals towards the drug and the improvement in general condition which usually follows its administration.

A second animal which had relapsed from a single dose of 0.6 gm. was given five doses of 0.5 gm. and three doses of 0.6 gm. in 18 days, but the treatment was ineffectual and relapse occurred 1 week after it was discontinued.

In a third rabbit, the effect of the treatment was uncertain. This was likewise a case of relapse from a single dose of 0.6 gm. per kilo. The animal was in poor condition when relapse occurred and retreatment was instituted. Two doses of 0.5 gm. per kilo were given with 48 hours intervening. The signs of trypanosomiasis were but slightly improved, and the animal was weaker, so the dose of drug was reduced to 0.3 gm. and two doses of this size were administered. Death occurred 3 days later without complete disappearance of the manifestations of trypanosomiasis.

By comparing these results with those previously reported from the treatment of rabbits infected with *Tr. gambiense*, it is at once apparent that the *rhodesiense* infections were much more difficult to influence. Results comparable with those obtained with single doses of 0.6 to 0.75 gm. per kilo could be obtained in *gambiense* infections with approximately one-third of this amount of drug, and while cures were uniformly obtainable in *gambiense* infections with from 0.3 to 0.35 gm. per kilo, there appeared to be no single dose of the drug with which this could be accomplished with safety in cases of *rhodesiense* infection.

CONCLUSIONS.

With the three classes of animal infections studied, the trypanocidal action of tryparsamide was found to be much less for *Tr. rhodesiense* than for *Tr. gambiense*, and a correspondingly greater difficulty was experienced in the treatment of the chronic tissue infections of *Tr. rhodesiense*.

This is, of course, entirely in accord with past experience in the treatment of human cases of trypanosomiasis due to the two organisms. It is still possible that something may be accomplished by the use of tryparsamide in cases of Rhodesian sleeping sickness on account of the tolerance exhibited to the drug and the possibility of employing an intensive system of treatment. Much less is to be expected, however, than in cases of infection due to *Tr. gambiense*.

SUMMARY.

A series of experiments was carried out to determine the effects of tryparsamide upon the infections produced in various species of animals by *Tr. rhodesiense*.

The strain of trypanosome used was one which possessed a very low virulence for guinea pigs, was fairly virulent for mice and rats, and highly virulent for rabbits.

Therapeutic experiments carried out on 24 hour infections in mice and rats showed that cures could be obtained in this class of infections by the administration of a single large dose of the drug amounting to approximately two-thirds of the maximum tolerable dose, as contrasted with similar results in cases of *gambiense* infections from approximately one-ninth and one-fifth of this dose respectively.

With advanced infections in rabbits, there appeared to be no single dose of the drug capable of insuring a cure which could be administered with safety, although some cures were obtainable with doses approximating the maximum tolerable dose. Treatment of these infections could be carried to a successful conclusion, however, by an intensive system of treatment in which large doses of the drug were administered at short intervals of time, and even relapses yielded to this treatment in some instances. The employment of such a method of treatment was possible on account of the unusual tolerance exhibited by animals to this drug, a fact which was previously emphasized.

These facts indicate that the outlook for the drug in the therapy of Rhodesian sleeping sickness is much less hopeful than in *gambiense* infections, though it is felt that some benefits may be derived from its use.

BIBLIOGRAPHY.

1. Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1919, xxx, 437, 455.
2. Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

STUDIES OF THE ACID-BASE EQUILIBRIUM IN DISEASE FROM THE POINT OF VIEW OF BLOOD GASES.*

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(Received for publication, August 2, 1920.)

INTRODUCTION.

The problem of acidosis resolves itself into two fundamental questions. First, in any given case is there an acidosis in the sense that the available alkali of the blood is diminished through an increase in non-volatile acid; and, second, is there an acidosis in the sense of an actual change in blood reaction in the acid direction, of an increase, in other words, of the concentration of ionized hydrogen? In disease these conditions may occur alone or combined. To recognize each and to distinguish between them is of some clinical importance. Data concerning the former are abundant in the literature; concerning the latter, somewhat less so; and data showing the two simultaneously are few.

The question of available alkali of the blood in disease has been approached by a large number of investigators and by divergent methods. Of the more recent work the following general types may be cited by way of illustration: (*a*) determination of titratable alkalinity of the blood by the method of Sellards (1); (*b*) determination of the carbon dioxide capacity of the serum by the methods of Van Slyke (2); (*c*) determination of the oxygen-combining power of the blood at a given tension of oxygen as done by Barcroft (3).

The question of the actual reaction of the blood in health and disease has also been approached in several ways. The reaction can be measured directly either electrometrically by the so called gas chain method of Hasselbalch (4) or by the use of indicators as employed by Sörensen (5) and more recently by Bayliss (6). It can also be arrived at indirectly from the behavior of the oxygen dissociation curve of the blood, the determination of the degree of meionexy of Barcroft (3).

* This paper is the first of a series of studies of the physiology and pathology of the blood from the Harvard Medical School and allied hospitals.

Finally it may be calculated from the carbon dioxide dissociation curve provided that the arterial carbon dioxide tension or concentration is known. Hasselbalch (7) has published a formula for such calculation which is dependent upon the principle shown by L. J. Henderson (8) that the reaction of the blood is dependent chiefly upon the ratio of free carbonic acid to bicarbonate, which can be expressed by the formula

$$[\overset{+}{H}] = K \frac{[H_2CO_3]}{[BHCO_3]}.$$

in which $[\overset{+}{H}]$ is the concentration of ionized hydrogen, $[H_2CO_3]$ the concentration of free carbonic acid and $[BHCO_3]$ the concentration of bicarbonates, and K a constant. The fraction $\frac{[H_2CO_3]}{[BHCO_3]}$ ordinarily has a value of approximately $\frac{1}{20}$. Changes in this ratio, it can easily be seen, will denote changes in the $[\overset{+}{H}]$, that is in blood reaction, while changes in the actual magnitude of the denominator will denote changes in available alkali of the blood and changes in the numerator changes in carbon dioxide tension. Knowledge, then, of the two terms of the ratio in any given blood will tell us the two most fundamental facts of acidosis.

For clinical purposes the simplest way of arriving at the value of the ratio and of determining the actual magnitude of the two terms of the fraction is to secure the data necessary to plot the so called carbon dioxide diagram of the blood of Haggard and Y. Henderson (9). This diagram, which incidentally is essentially a graphic representation of L. J. Henderson's formula, consists in the carbon dioxide dissociation curve of the blood determined after the manner of Christiansen, Douglas, and Haldane (10) with a mark upon it which denotes its intersection with the coordinate representing the tension or concentration of carbon dioxide in the arterial blood, this point being called the A-point.

After a conversation with Professor Y. Henderson in January, 1920, we were greatly impressed with the lucidity of such diagrams in the interpretation of problems of acid-base equilibrium, and as a result we undertook the present research which consists in the construction of such diagrams for the blood of patients, for the purpose of determining the presence or absence of non-volatile acid in their blood, and of actual changes in their blood reaction.

The information to be gathered from the dissociation curve thus plotted with its A-point is as follows: In the first place the abscissæ in the diagram indicate the tension of carbon dioxide in the blood and therefore the concentration of dissolved carbonic acid, for it is well known that the concentration of carbonic acid in physical solution is directly proportional to its partial pressure, or tension. The abscissa

therefore is the numerator of the fraction in L. J. Henderson's formula [H_2CO_3]. Second, the ordinates are indices of the denominator of the fraction, or the concentration of bicarbonates in the blood [$BHCO_3$]. For although what is actually determined is the total carbonic acid of the blood, not only in chemical combination as bicarbonate, but in physical solution as well, yet, since the latter is directly proportional to the tension, it can be disregarded. Indeed Haggard and Henderson in their diagram by drawing a diagonal base-line show directly the amount of carbon dioxide in physical solution and chemically combined. We have not used this method, as in the majority of curves in the literature the total carbonic acid at any given tension has usually been plotted above a horizontal base-line.

If now the abscissa represents dissolved carbonic acid concentration and the ordinate bicarbonate concentration, and if hydrogen ion concentration is dependent upon the ratio of these two to each other multiplied by a constant, then for any given point in the diagram a series of radii drawn through the zero point will indicate the magnitude of the hydrogen ion concentration. Also in any given arterial blood the position of the A-point will show the reaction. As long as the value of the ratio [H_2CO_3] : [$BHCO_3$] remains constant, though its actual terms may vary in magnitude, the A-point will always fall on the same straight line drawn through the zero point. We have therefore drawn a diagonal, the line 0C in Text-fig. 1, which runs approximately through the A-points of a number of normal bloods. Pathological bloods, if the A-points fall to the left of this line, will have a more alkaline, if to the right a less alkaline reaction than normal.

By the carbon dioxide diagram then we are informed, first, of the presence or the absence of acidosis in the sense of a changed blood reaction, and, second, of the presence or absence of non-volatile acid in the blood. This latter is shown by the level of the dissociation curve. The effect of adding acid to the blood is to diminish its available alkali. This means that the dissociation curve will be moved downward, for the ordinate represents the concentration of blood bicarbonate. The two main facts of acidosis then are revealed by the diagram, through the position of the A-point and the level of the curve.

Methods.

There is nothing new in the methods we have made use of. Blood was drawn from an arm vein and placed in a tube containing some dry oxalate crystals. About 5 cc. were placed in each of three tonometers which had previously been filled with mixtures of air and carbon dioxide of known tension. The type of tonometer[1] used was that shown by Barcroft (3). The tonometers were then placed in a brass cylinder filled with water at body temperature and slowly rotated in a horizontal position for 10 minutes by a motor.[2] After equilibration the carbon dioxide content of the blood from each tonometer was determined by means of Henderson and Smith's blood gas apparatus (11).

Three points in the dissociation curve were thus obtained and plotted as in Text-fig. 1. The ordinates represent the concentration of carbon dioxide of the blood in terms of volumes per cent; that is, the content as found by blood gas analysis. The abscissæ represent the tension of carbon dioxide in millimeters of mercury; that is, the partial pressure of carbon dioxide in the equilibrating atmosphere. These points having been obtained for any given blood, the most probable curve was then drawn through them.

The next step in the construction of the carbon dioxide diagram is the fixing of the arterial or A-point. This can be done, of course, by finding either the concentration of carbon dioxide in the arterial blood, its appropriate abscissa, and the point where this intersects the curve, or by determining the tension of carbon dioxide in the arterial blood and finding the point where its ordinate intersects the curve. If both methods were used they would serve as a check upon each other.

The concentration of carbon dioxide in arterial blood we obtained by drawing samples under oil from the radial or brachial artery after the method of Stadie (12), and then determining their carbon dioxide content with the Henderson apparatus. Direct determination of the tension of gases in the circulating blood of animals has been done experimentally by Krogh and Krogh (13), but could hardly be done

[1] Barcroft (3), Fig. 145.

[2] This rotating bath was a copy of one which was in use in Barcroft's laboratory at Cambridge in 1913.

satisfactorily in man. In the absence of lesions of the lungs or of disturbances of the circulation through the pulmonary circuit, however, it is safe to assume that the arterial blood, as far as carbon dioxide is concerned, is essentially in tension equilibrium with the alveolar air, as obtained by Haldane and Priestley's method (14).

In plotting the A-point of our several curves we sometimes have made use of the carbon dioxide tension of the alveolar air, and sometimes of the carbon dioxide content of the arterial blood. The method used in each instance is noted in Table I.

Cases Studied.

We determined, in all, fifteen dissociation curves. All the points determined are shown in Table I, while the actual curves with their A-points are shown in Text-fig. 1. Two of these curves were of the blood of normal individuals, three of a diabetic with acidosis, two of a nephritic with acidosis, three of pneumonia patients, two of anemia (one primary and one secondary), one of cerebral hemorrhage, one of myelogenous leucemia, and one of idiopathic tetany.

Normal Individuals.—As we stated earlier in interpreting the carbon dioxide diagram one phase of the question is answered by the level of the dissociation curve. Since this is true, it becomes necessary to discover the level of the normal curve. We have searched the literature for such curves and found several. In Text-fig. 1 a shaded zone is shown. This zone contains curves of the blood taken from the work of Christiansen, Douglas, and Haldane (10), of Hasselbalch (7), of Parsons (15), of Joffe and Poulton (16), of Liljestrand and Lindhard (17), and also curves of two cases described in the present paper, Cases A and B (Curves 1 and 2). A similar zone has been published by Straub and Meier (18), which is said to include 320 points of the bloods of 64 normal persons. The lower border of their zone falls within our zone, but their upper border is very much higher and so at variance with the work of other investigators that we are inclined to believe it incorrect. All the A-points of the blood of Cases A and B and of those determined by Christiansen, Douglas, and Haldane, and Liljestrand and Lindhard fall either on the 0C line in Text-fig. 1, or at a distance on either side of it so slight that it would

TABLE I.

Dissociation Curve; Points Actually Determined.

Curve No.	Case.	Condition.	CO_2 tension.	CO_2 content.	A-point.
			mm.	*vol. per cent*	*mm.*
1	A	Normal.	16.8	38.0	37.5
			41.0	51.0	
			72.0	61.0	
2	B	"	17.6	37.0	40.6
			36.0	49.0	
			65.0	59.0	
3	C	Diabetic acidosis.	19.5	19.0	14.6
			42.5	27.0	
			74.0	39.5	
4	C	Same. 1 day later.	18.1	21.0	20.5
			38.2	30.0	
			78.2	39.0	
5	C	" 3 days after Curve 4 was determined.	18.5	Lost.	26.8
			42.5	41.0	
			67.0	48.0	
					per cent
6	D	Renal acidosis.	17.9	13.0	12
			32.5	22.0	
			67.0	36.0	
7	D	Same. Day after alkali administration.	18.5	45.0	60
			35.4	62.0	
			69.5	72.0	
8	E	Influenza pneumonia.	19.7	38.0	57
			31.5	44.0	
			68.0	61.0	
9	F	Lobar pneumonia.	18.5	34.0	52
			32.5	46.0	
			67.5	58.0	
10	G	Bronchopneumonia.	21.8	50.0	68
			39.4	58.0	
			69.5	70.0	

TABLE I—*Concluded.*

Curve No.	Case.	Condition.	CO_2 tension.	CO_2 content.	A-point.
			mm.	*vol. per cent*	*per cent*
11	H	Cerebral hemorrhage.	22.0	46.0	56
			34.5	54.0	
			69.2	65.0	
					mm.
12	I	Myelogenous leucemia; secondary anemia.	18.9	47.0	40.6
			42.6	62.0	
			80.5	72.0	
13	J	Secondary anemia.	17.7	42.0	Lost.
			36.6	50.0	
			70.5	63.0	
					per cent
14	K	Idiopathic tetany.	18.8	43.0	58
			38.2	53.0	
			70.0	66.0	
15	L	Primary anemia.	19.5	55.0	70
			35.8	69.0	
			73.8	74.0	

make a difference of not more than 0.02 in the pH. The data for placing the A-point of the other curves taken from the literature were not found in the several papers.

Diabetic Acidosis.—Three curves (Nos. 3, 4, and 5) were obtained from a case of diabetic acidosis (Case C).

The first of these curves (No. 3) was obtained on January 23, 1920. The patient had then just entered the hospital with a moderately severe diabetes mellitus, and with a considerable acetonemia. He was somewhat drowsy but not in coma. There was no noticeable hyperpnea and no subjective dyspnea. The low position of the curve on this day shows a very low combining power of the blood with carbon dioxide and hence indicates the presence of a large quantity of non-volatile acid in the blood. Nevertheless, the A-point of this curve is practically on the 0C line, which means presumably that his blood reaction was normal. He had, in other words, what has been called by Y. Henderson and others a compensated acidosis. He had acidosis

in the sense of having abnormal acid present in his blood, but he had by increasing his pulmonary ventilation and thereby reducing the tension of carbon dioxide in his alveolar air so reduced the dissolved carbon dioxide of his blood that the normal value of the $[H_2CO_3]$: $[BHCO_3]$ ratio was preserved. Through his pulmonary response he had kept his blood reaction normal.

After this he fasted, but was given water and salt freely, and on January 24 his dissociation curve had risen to the position of Curve 4. The A-point of this curve is slightly to the acid side of the 0C line and may indicate that his compensation was not quite so good as it had been the day before. It must be said, however, that as we approach the lower left-hand corner of the diagram the position of the A-point becomes less and less accurate. The experimental error in determining the carbon dioxide content by the method used is probably at least 1 per cent, and with low curves an error of 1 per cent will make much more change in the pH than it would with curves in the normal position. We should hesitate to say then that the A-point of Curve 4 really proved the presence of a decompensated acidosis. The fact that Curve 4 is higher in position than Curve 3 shows that the patient had somewhat improved his supply of available blood alkali.

On January 27, Curve 5 was obtained. This shows a very striking increase in available alkali and an A-point on the 0C line, indicating perfect compensation.

Unfortunately no further observations were obtained with this patient, for he left the hospital against advice.

Nephritic Acidosis.—We also had an opportunity to obtain two curves in the case of a man, 55 years old (Case D), who was brought to the hospital with an intense grade of hyperpnea. It proved to be impossible to measure his pulmonary ventilation, but from the general character of his breathing as compared with a similar case of hyperpnea reported by Means and Rogers (19) it seemed probable that he was breathing 40 to 50 liters per minute.

His underlying malady was arteriosclerotic nephritis but he had in addition an obstructing prostate and a double pyelonephritis. He was in a profound state of uremia and retention acidosis. The dissociation curve (Curve 6) obtained the day he came in, February 19, 1920,

had the lowest position of any in the series, indicating that the available alkali was very greatly reduced. Moreover, the position of the A-point of the curve definitely to the right of the 0C line shows that, in spite of the extreme hyperpnea, the respiratory response was insufficient to preserve the normal $[H_2CO_3] : [BHCO_3]$ ratio and he had an actual change in the reaction of his blood in the acid direction, a decompensated acidosis in other words. The actual shift in the pH, as explained earlier, in this part of the diagram can only be roughly estimated, but it could not have been less than 0.2. His actual oxygen saturation on this day was 89.6 per cent and his hemoglobin 69.3 per cent (calculated from the oxygen capacity).

After the blood had been drawn for the first curve he was promptly started on large amounts of sodium bicarbonate intravenously and by rectum. A free flow of urine was also obtained by catheterization.

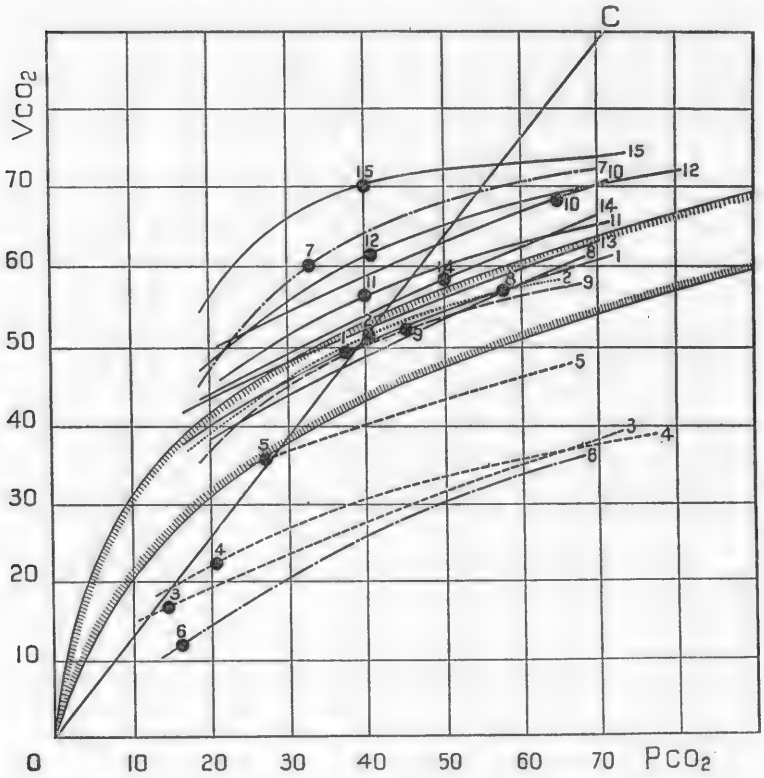
The next day, after he had had approximately 175 gm. of sodium bicarbonate, there was a most extraordinary change. The hyperpnea had entirely gone and he was no longer drowsy. Curve 7 was obtained. The change in the curve is as remarkable as that in the clinical condition. From being very low it had moved to a level higher than normal. On that day, then, the available alkali was greater than in normal individuals. Furthermore, the A-point had moved to the left side of the 0C line to such a distance that he probably had, on that day, an actual shift in blood reaction in the alkaline direction. A large amount of fluid had been administered to him and this was reflected in the hemoglobin, which had dropped to 51.1 per cent. Moreover, he no longer had arterial anoxemia, his arterial blood being 93.5 per cent saturated.

The improvement, of course, was transient, as he had practically no renal tissue. He became uremic and acidotic again, and died on February 23.

Pneumonia.—A curve was obtained from each of three patients with pneumonia.

The first of these (Curve 8) was that of the blood of a man, 39 years old, with influenza pneumonia (Case E). The curve was obtained on February 18, 1920. The influenza began on February 12 and the pneumonia on February 16. It was of the fulminating variety of influenza pneumonia. On the day the curve was obtained there were

scattered areas of consolidation throughout both lungs. There were frothy bloody expectoration and a marked diffuse bronchitis. Respirations were rapid and shallow. He had a dull, leaden cyanosis of the mucous membranes and finger tips. The striking point about the diagram of this case is that while the level of the curve is quite within the normal zone, the A-point is far to the right of the 0C line, indicating



TEXT-FIG. 1. Carbon dioxide diagram in health and disease. The ordinates represent carbon dioxide content of the blood in volumes per cent (V CO_2), the abscissæ, tension of carbon dioxide in millimeters of mercury (PCO_2). The shaded zone indicates the area within which we believe the curves of normal blood fall. The line 0C represents the hydrogen ion concentration of normal blood. The arterial or A-points are shown by black dots. The points in the several curves actually determined, and also the condition of the patients, are shown in Table I.

a definite shift in the blood reaction in the acid direction, equal roughly to a decrease of about 0.10 in the pH. On the same day the oxygen saturation of the arterial blood of this case was 59.3 per cent and the hemoglobin 103.8 per cent. The arterial blood as drawn into a syringe was quite as dark as normal venous blood. He died on February 19. No autopsy was obtained.

The next diagram of pneumonia blood (Curve 9) is that of a man, 59 years old, with true pneumococcus (Type IIa) lobar pneumonia (Case F). The curve was obtained on February 11, 1920, the pneumonia having begun about 2 weeks before. He had on this day extensive double lobar pneumonia, the course of which had been progressively worse. His breathing was labored and grunting and he had marked cyanosis. About 8 hours after the blood for the diagram was taken he died. No autopsy was obtained. The arterial oxygen saturation on February 11 was 74.5 per cent and the hemoglobin 118 per cent. This curve, like the preceding one, is within the normal zone, the A-point also being to the right of the 0C line, but less strikingly so than with the blood of Case E.

The last pneumonia blood studied (Curve 10) was that of a man, 43 years old, with postoperative bronchopneumonia. He also had pulmonary emphysema and chronic bronchitis (Case G). He was operated on for gall stones on February 21, 1920, and on February 22 the pneumonia began. The curve was obtained on February 25. On that day there were areas of consolidation scattered through both lungs, many râles, deep cyanosis, and rapid grunting breathing. It is interesting to note that the level of the dissociation curve is above the normal zone, yet the A-point like that of Curve 8 is well to the right of the 0C line. The arterial oxygen saturation on February 25 was 86.3 per cent and the hemoglobin 122 per cent. He made an uneventful recovery.

Blood Disease.—The curves for the blood of three patients with blood disease were obtained. One of these (Curve 12) was that of a man, 33 years old, in an aleucemic phase of myelogenous leucemia; he likewise had a marked secondary anemia. The red cell count was 1,280,000, the white cell count 5,600, and the hemoglobin 45 per cent. The interesting thing about his curve is that it lies throughout at a higher level than the upper border of the normal zone and that the A-point lies to the left of the 0C line.

Curve 13 of a man, 44 years old, with secondary anemia of moderate grade (Case J) whose red cell count was 3,248,000, white cell count 6,600, and hemoglobin 73 per cent also lay at a higher level than normal. Data for plotting the A-point of this blood were unfortunately not obtained.

The diagram of the blood of a man, 56 years old (Case L), with pernicious anemia (Curve 15) showed a still higher position of the dissociation curve and an A-point still further to the left than that of Curve 12. The red cell count was 1,849,000, the white cell count 4,000, and the hemoglobin 25 per cent.

Miscellaneous Curves.—One diagram (Curve 11) was obtained from a man, 58 years old, with a cerebral hemorrhage (Case H). This showed a slightly higher level of the curve than did the normal curves, and an A-point slightly to the left of the 0C line.

The blood of a woman, 28 years old, with idiopathic tetany (Case K) showed a curve just above the upper border of the normal zone (Curve 14), and an A-point slightly to the right of the 0C line.

DISCUSSION.

The interpretation of these results presents considerable difficulty. As we pointed out earlier, even the range of variation of the level of the carbon dioxide dissociation curve of normal blood is not known. The zone we have shown in Text-fig. 1, while it includes the curves determined for normal blood by a number of different observers, may perhaps not represent the true range. It may be either too wide or too narrow, or either or both of its borders may be too high or too low. It will require a large series of normal curves to determine correctly the limits of normal variation. Nevertheless, it seems to us that the striking variations from the few normal curves available, shown by some of the pathological bloods here studied, are not without clinical interest.

Before discussing these variations, we shall consider some of the possible sources of error. In the first place, what are the relative degrees of accuracy of the several procedures involved in the construction of the carbon dioxide diagrams? The shape and level of the dissociation curves we believe are substantially correct. The concentration

of carbon dioxide in the blood by the method used should be accurate within 1 volume per cent; that of carbon dioxide tension should certainly be accurate within less than 1 mm.

The position of the 0C line which we have drawn to represent the normal ratio of free carbonic acid to bicarbonate according to the calculations of Straub and Meier from Hasselbalch's formula would indicate a pH of approximately 7.35. The actual pH of arterial blood as found by the gas chain method is given by Michaelis (20) as 7.45. As already stated, the A-point of the curves of the blood determinations in cases described by Haldane, Liljestrand, and Lindhard, and in Cases A and B of the present paper falls either on the line, as we have drawn it, or at an insignificant distance from it.

About the accuracy of the position of the A-points of the several curves we must speak with less assurance. As has already been said, two ways of plotting this point were used. Both these methods are open to criticism. The plotting of the A-point from the carbon dioxide tension of the alveolar air would be satisfactory provided, first, that accurate samples of arterial pulmonary air could always be secured, and, second, that the arterial blood is always in tension equilibrium with the alveolar air. In normal individuals both these provisions hold true. It is undoubtedly possible to obtain accurate alveolar carbon dioxide tension by Haldane's method in well trained normal subjects. So also it is entirely probable that their arterial blood is in tension equilibrium with their alveolar air, as far as carbon dioxide is concerned. In the sick patient, however, not only may it be impossible to obtain reliable Haldane samples of arterial pulmonary air, but furthermore, if there is a gross pathological condition within the thorax, it is quite possible that alveolar air and arterial blood will not be in tension equilibrium.

It was for this reason that in patients with pulmonary disease, or with those from whom for any reason we could not get good alveolar air samples, we resorted to the method of plotting the A-point from the carbon dioxide content of the arterial blood. This method might at first be thought to be free from the errors of the alveolar air method, but yet, as Peters (21) has recently pointed out to us, it is not entirely so. Provided the patient made no alteration in his pulmonary ventilation while the arterial sample was being drawn, there would be no

error, but if he either increased or decreased his ventilation, then we should have an abnormal carbon dioxide content, abnormally low in the former case, abnormally high in the latter, which would give us a false position of the A-point, too far to the left in the former case, too far to the right in the latter.

Another type of possible error is due to the nature of the diagram itself. In places where the dissociation curve is more nearly horizontal than vertical an A-point plotted from the carbon dioxide tension will obviously be more accurate than one plotted from the carbon dioxide content; and conversely, when the curve is more nearly vertical than horizontal, the carbon dioxide content will give the more accurate point. To illustrate this principle take for example the A-point of Curve 15. Here an increase of 1 volume per cent in carbon dioxide content would move the A-point some three times nearer the 0C line than would a rise of 1 mm. in the tension, and a rise of 2.5 volumes per cent in the content would place it on the 0C line, while a rise of 2.5 mm. in tension would still leave it far to the left.

One last source of error must be mentioned, and that is the effect of oxygenation upon the level of the carbon dioxide dissociation curve. Christiansen, Douglas, and Haldane (10) in their original paper showed that the curve for oxygenated blood has a definitely lower level than that of reduced blood. The curves that we have included in our normal zone were all those of oxygenated blood. Since arterial blood is normally nearly saturated no error is introduced in the plotting of the A-point by the effect of oxygenation on the carbon dioxide capacity except with such patients as have an arterial anoxemia. It will be noted that all three pneumonia patients studied had arterial anoxemia. It might be argued that in these cases it was not legitimate for us to place an A-point from the carbon dioxide content of their anoxemic arterial blood upon a dissociation curve of fully oxygenated blood. This objection would hold except for the interesting fact just discovered by Y. Henderson that the dissociation curve of oxalated blood (that has not been exposed to air) usually shows no shift with oxygenation (22). The shift described by Christiansen, Douglas, and Haldane occurred in defibrinated blood. This finding of Y. Henderson has, we understand, been confirmed by L. J. Henderson (23). Its explanation is yet to be discovered, but

it enables us in the present research to eliminate the effect of oxygenation and reduction, and therefore to plot our A-points upon a curve of fully oxygenated blood, even though they are plotted from carbon dioxide contents of anoxemic bloods.

We shall now discuss the interpretation of our diagrams. We might repeat that our main object is twofold: first, to discover acidosis in the sense of diminished alkali reserve as shown by the level of the curve, and, second, to discover acidosis in the sense of altered blood reaction as shown by the position of the A-point; to distinguish, in other words, between acidosis compensated and acidosis decompensated, and of course to discover the opposite conditions of compensated and decompensated alkalosis if such exist.

The curves obtained with the diabetic patient (Curves 3, 4, and 5) show nothing particularly new. They illustrate clearly a condition of marked but compensated acidosis; that is, great reduction in available alkali but perfect pulmonary response with maintenance of normal blood reaction, and recovery therefrom.

The curves obtained with the nephritic case are even more striking. When the first curve (Curve 6) was obtained the patient undoubtedly was suffering from a decompensated acidosis. The change in the position of the curve over night, chiefly due no doubt to the alkali he had received, is very remarkable. From a condition of decompensated acidosis he had passed really in this brief interval to one of decompensated alkalosis, to use the same terminology.

The curves of the three pneumonia patients are perhaps more illuminating. Two of them (Curves 8 and 9) are within the normal zone, showing that both these patients had a normal reserve of alkali and therefore presumably no abnormal acid in the blood. With both of them the A-point is displaced in the acid direction. The curve of the third pneumonia patient (Case G) is at a higher level than normal, but like the other two its A-point is displaced to the right. These A-points are plotted from the carbon dioxide content of the arterial blood. As to their accuracy, we have already pointed out the effect of over or under ventilation. We have no proof that the ventilation of these patients was unaltered while we drew the arterial blood samples. However, they were all very ill, and took little or no interest in the procedure. Cocainization prevented pain, and therefore there

seems good reason to believe that no alteration in breathing occurred. There was no holding of the breath and no decrease in rate of breathing, none at least that could be told by observation. If any change occurred then it was more likely an increase than a decrease. But an increase would move the A-point to the left, which is the opposite to what was actually found. All things considered then, we feel that it is reasonably certain that the hydrogen ion concentration of the pneumonia blood was really altered in the acid direction.

In pneumonia then we may have in critically ill patients a condition in which although the buffer of the blood is quite normal, the reaction is more nearly acid than normally. There is an acidosis in the latter sense, but none in the former. The only explanation of this rather paradoxical state of affairs that occurs to us is that the condition actually is one of carbonic acidosis. A person with no non-volatile acid present in the blood, but with a pulmonary ventilation insufficient to preserve the normal $[H_2CO_3] : [BHCO_3]$ ratio would have just such a diagram as these pneumonia patients. We already know because of their arterial anoxemia that they are not getting oxygen properly into their blood. It is certainly conceivable that in a similar way they are not getting carbon dioxide out. Their effective lung ventilation may be much greater than normal and still be insufficient, and it is not difficult to see how, because of mechanical limitations to the respiratory movements from pleurisy or through consolidation of the lungs, or because of decreased permeability of the alveolar membrane, the output of carbon dioxide might be hindered.

Henderson and Haggard (24) have shown that the reaction to increased carbon dioxide is an increase in the carbon dioxide capacity. This they observed in experimental morphine poisoning. It is the converse of the reaction to non-volatile acidosis. In the one case the available alkali being reduced, the tension of carbon dioxide is reduced in compensation, while in the other case the tension being increased the alkali is increased in compensation. It occurred to us that this might be what was happening in Case G (Curve 10). This curve is at a higher level than normal, yet the A-point is well to the right of the 0C line. The reaction of the blood then is more nearly acid than it should be, yet the available alkali is greater than normal also. This may be an attempt at compensation, and it can be seen that if the car-

bon dioxide capacity of this blood increased only 3 volumes per cent more, the A-point would fall on the 0C line. It is probably only a coincidence, but it is interesting to note that this attempt at the compensation of a carbonic acidosis in this particular case proved a good prognostic sign, for this patient recovered shortly afterwards, while the other two died.

Of the curves of the three cases of blood disease (Curves 12, 13, and 15), two showed a higher level than normal, that of Case L with primary anemia being in the highest position of all the curves obtained. The curve of Case I with myelogenous leucemia in an aleucemic stage and with a marked secondary anemia, was also at a higher level than normal. The A-points of these two curves are also to the left of the 0C line, denoting an increased alkalinity of the blood. The curve of the blood of the other anemic patient (Case J) is within the normal zone; the A-point of this curve was not obtained.

What may be the significance of the high level of these curves in anemia we do not know. Peters (21) tells us that he has found much the same thing in some of the anemic blood he has studied. The dissociation curve of the blood plasma lies at a higher level than does that of whole blood as shown by Hasselbalch (7) and by Joffe and Poulton (16), and it may be that the levels of anemic blood are higher in part because of the greater proportion of plasma that they contain. For the position of the A-points of these curves we also can offer no explanation. Both Curves 12 and 15 are more nearly horizontal than vertical near the 0C line. Hence an A-point plotted from tension would be more accurate than one plotted from content. That of No. 12 was plotted from tension and is, we believe, reasonably accurate. That of No. 15 was plotted from content.

The two remaining curves are those of Case K with idiopathic tetany (Curve 14) and of Case H with cerebral hemorrhage (Curve 11). The former was constructed to see whether any evidence of a condition of alkalosis could be found. None was found, for the level of the curve is just above the upper limit of the normal zone and the A-point on the right, not on the left, of the 0C line. The curve of Case H is, on the other hand, a little high and the A-point slightly to the left. The single observation in tetany does not prove very much for, as has been shown by Wilson, Stearns, and Thurlow (25), there are marked

and rapid variations in the acid-base equilibrium in tetany, and it is possible that our patient may have been changing from a stage of alkalosis to one of acidosis at the time the blood was drawn.

Considering the A-points of the entire series, we find that they are so distributed as to indicate a range of variation in the pH of roughly from 7.25 to 7.55. Sonne and Jarlöv (26) calculating in a similar way found a range of variation from 7.15 to 7.40, the difference in the pH of our extremes thus being 0.30 and of theirs 0.25. Their highest hydrogen ion concentration was in a case of severe diabetes mellitus with acetonuria, and their lowest was in a case of chronic nephritis with hypertension. With the gas chain method Michaelis (20) has found variations from 7.74 to 7.12, the first being in a case of syphilitic hemiplegia, the second in a case of diabetic coma just before death. It seems entirely likely then that our A-points are all within a range of variation compatible with life, and that they are not necessarily artifacts.

As to therapeutic indications brought out by the present study, there are one or two that are worth mentioning, in the first place in regard to acidosis such as that presented by the diabetic (Case C) and the nephritic (Case D). While the diminution of alkali was of somewhat the same order of magnitude, the acidosis of Case C was apparently compensated, while that of Case D was decompensated. It is also of interest that while Case C had no appreciable amount of respiratory distress Case D had a maximum amount. This we believe may in part have been due to the fact that the latter was a case of decompensated, while the former was one of compensated acidosis. This brings up the problem of what are the indications for alkali therapy. The curve of Case C went back towards normal with no alkali administration in a perfectly satisfactory way, and indeed it is generally conceded now that alkali is usually unnecessary in the treatment of diabetic acidosis.

In compensated acidosis the return to a normal level will be accomplished by the elimination of neutralized acid by the kidney. The lungs have already done their part in preserving blood reaction. Under the circumstances it is questionable whether the giving of alkali will accomplish any great good, and indeed it is possible that if too much is given it may do harm by producing alkalosis. The indica-

tion then in compensated acidosis would seem to be rather to promote elimination by the kidneys by giving fluids freely, and, if, as in diabetes, this is coupled with fasting which would lessen the further production of non-volatile acid, it would seem to be sufficient therapy.

In decompensated acidosis, on the other hand, alkali administration is probably indicated, because here there is an insufficient pulmonary response. The supply of additional bicarbonate may well restore the $[H_2CO_3] : [BHCO_3]$ ratio to a normal value; indeed with Case D it did more than that and actually pushed his reaction to the other extreme. That it is desirable to restore blood reaction to normal cannot be doubted. All the high hydrogen ion concentrations have been found in critically ill patients, who, in fact, were usually in a moribund state. On the other hand, it is undoubtedly quite as important not to overdo the alkali therapy, as was done with Case D. A warning that excessive alkali therapy may be harmful has been given by Allen, Stillman, and Fitz (27). They believe, particularly in persons with damaged kidneys, that alkali should be used with great caution. That tetany can be produced after alkali administration is in itself evidence that alkalosis as well as acidosis may be harmful *per se* and equally to be avoided. The case reported by Harrop (28) of a woman with mercuric chloride poisoning who developed tetany and showed a plasma bicarbonate capacity of 80 volumes per cent after the intravenous injection of 60 gm. of sodium bicarbonate is particularly interesting in this regard. Equally pertinent is the recent work of Grant and Goldman (29), who succeeded in producing tetany and other unpleasant symptoms in themselves by prolonged voluntary forced breathing. They were able at the same time to demonstrate a change in the pH of the blood in the alkaline direction, and had the interesting situation of a low position of the dissociation curve as shown by the alveolar carbon dioxide tension and the carbon dioxide capacity of the plasma, and at the same time a blood more alkaline in reaction than normally. It really is the diametrically opposite condition to that shown by our pneumonia patient, Case G (Curve 10).

As to the indications for treatment in pneumonia, we can only say that they would seem to be to facilitate lung ventilation. According to our present knowledge, however, such indications can only be indirectly met. Measures which facilitate the mechanics of respiration,

the relief of pleural pain, the placing of the patient in the upright position, are of this order. So too are specific or other forms of treatment which by reducing fever decrease the metabolism, and hence the carbon dioxide output, and so decrease the amount of ventilation necessary to preserve the normal acid-base ratio. As Hoover (30) has pointed out, anoxemia may or may not be relieved by oxygen administration, but in any event difficulties in carbon dioxide elimination cannot be so relieved, and consequently it seems unlikely that oxygen administration will have any great beneficial effect in the treatment of pneumonia. Several years ago Newburgh, Means, and Porter (31) showed that the respiratory center gradually fails during the course of experimental pneumonia. It has also been shown by Davies, Haldane, and Priestley (32) that resistance to breathing actually causes fatigue of the center. It is possible that this failure was in part due to such fatigue, the fatigue resulting from an incompletely successful respiratory effort to overcome a tendency towards carbonic acidosis.

SUMMARY.

Carbon dioxide diagrams (Haggard and Henderson (9)) have been constructed for the blood of a series of hospital patients as a method of studying disturbances in their acid-base equilibrium.

A diabetic with a low level of blood alkali, but with a normal blood reaction, a compensated acidosis in other words, showed a rapid return towards normal with no treatment but fasting and increased water and salt intake.

A nephritic with a decompensated acidosis and a very low blood alkali was rapidly brought to a condition of decompensated alkalosis with a high blood alkali by the therapeutic administration of sodium bicarbonate.

It is suggested that the therapeutic use of alkali in acidosis is probably only indicated in the decompensated variety, and that there it should be controlled carefully and the production of alkalosis avoided.

The diagram obtained in three pneumonia patients suggested that they were suffering from a condition of carbonic acidosis, due perhaps to insufficient pulmonary ventilation.

In two out of three cases of anemia the dissociation curve was found to lie at a higher level than normal. No explanation for this finding was offered.

BIBLIOGRAPHY.

1. Sellards, A. W., A clinical method for studying titratable alkalinity of the blood and its application to acidosis, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 101.
2. Van Slyke, D. D., Studies of acidosis. II. A method for the determination of carbon dioxide and carbonates in solution, *J. Biol. Chem.*, 1917, xxx, 347.
3. Barcroft, J., The respiratory function of the blood, Cambridge, 1914.
4. Hasselbalch, K. A., Elektrometrische Reaktionsbestimmung kohlensäurehaltiger Flüssigkeiten, *Biochem. Z.*, 1911, xxx, 317.
5. Sörensen, S. P. L., Über die Messung und Bedeutung der Wasserstoffionenkonzentration bei biologischen Prozessen, *Ergebn. Physiol.*, 1912, xii, 393.
6. Bayliss, W. M., The neutrality of the blood, *J. Physiol.*, 1919–20, liii, 162.
7. Hasselbalch, K. A., Die Berechnung der Wasserstoffzahl des Blutes aus der freien und gebundenen Kohlensäure desselben, und die Sauerstoffbindung des Blutes als Funktion der Wasserstoffzahl, *Biochem. Z.*, 1916–17, lxxviii, 112.
8. Henderson, L. J., Das Gleichgewicht zwischen Basen und Säuren im tierischen Organismus, *Ergebn. Physiol.*, 1909, viii, 254.
9. Haggard, H. W., and Henderson, Y., Hemato-respiratory functions, *J. Biol. Chem.*, 1919, xxxix, 163.
10. Christiansen, J., Douglas, C. G., and Haldane, J. S., The absorption and dissociation of carbon dioxide by human blood, *J. Physiol.*, 1914, xlviii, 244.
11. Henderson, Y., and Smith, A. H., Applications of gas analysis. V. Blood gas analysis, *J. Biol. Chem.*, 1918, xxxiii, 39.
12. Stadie, W. C., The oxygen of the arterial and venous blood in pneumonia and its relation to cyanosis, *J. Exp. Med.*, 1919, xxx, 215.
13. Krogh, A., and Krogh, M., On the tensions of gases in the arterial blood, *Skand. Arch. Physiol.*, 1910, xxiii, 179.
14. Haldane, J. S., and Priestley, J. G., The regulation of the lung ventilation, *J. Physiol.*, 1904–05, xxxii, 225.
15. Parsons, T. R., The reaction and carbon dioxide carrying power of blood—a mathematical treatment. Part I, *J. Physiol.*, 1919–20, liii, 42.
16. Joffe, J., and Poulton, E. P., The CO_2 dissociation curves of human oxygenated and reduced blood and human plasma, *J. Physiol.*, 1919–20, liii, p. lxvi.
17. Liljestrand, G., and Lindhard, J., The determination of the circulation rate in man from the arterial and venous CO_2-tension and the CO_2-output, *J. Physiol.*, 1919–20, liii, 420.
18. Straub, H., and Meier, K., Die Bestimmung der Blutreaktion aus der Kohlensäurebindungskurve, *Deutsch. Arch. klin. Med.*, 1919, cxxix, 54.
19. Means, J. H., and Rogers, O. F., Jr., Observations upon a case of extreme acidosis occurring in a man with bilateral cystic kidneys, *Am. J. Med. Sc.*, 1917, cliii, 420.

20. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.
21. Peters, J. P., Jr., personal communication.
22. Henderson, Y., personal communication.
23. Henderson, L. J., personal communication.
24. Henderson, Y., and Haggard, H. W., I. High levels of CO_2 and alkali, *J. Biol. Chem.*, 1918, xxxiii, 333.
25. Wilson, D. W., Stearns, T., and Thurlow, M. DeG., The acid-base equilibria in the blood after parathyroidectomy, *J. Biol. Chem.*, 1915, xxiii, 89.
26. Sonne, C., and Jarlöv, E., Untersuchungen über die Wasserstoffionenkonzentration des Blutes bei verschiedenen Krankheiten, *Deutsch. Arch. klin. Med.*, 1918, cxxiv, 379.
27. Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, 561.
28. Harrop, G. A., Jr., The production of tetany by the intravenous infusion of sodium bicarbonate. Report of an adult case, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 62.
29. Grant, S. B., and Goldman, A., A study of forced respiration: experimental production of tetany, *Am. J. Physiol.*, 1920, lii, 209.
30. Hoover, C. F., Moisture in the air spaces of the lungs and oxygen therapy, *J. Am. Med. Assn.*, 1918, lxxi, 880.
31. Newburgh, L. H., Means, J. H., and Porter, W. T., Respiratory mechanism in pneumonia, *J. Exp. Med.*, 1916, xxiv, 583.
32. Davies, H. W., Haldane, J. S., and Priestley, J. G., The response to respiratory resistance, *J. Physiol.*, 1919–20, liii, 60.

RELATION BETWEEN THE VIRULENCE OF STREPTOCOCCI AND HEMOLYSIN.

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(Received for publication, September 28, 1920.)

Since the original observations on streptolysin by Marmorek (1) in 1902, there has been much discussion concerning the relation between the hemolytic property and the pathogenicity of streptococci. Clinical and laboratory studies have been made, but, due to the complexity of the problem, the opinions arrived at have been contradictory. In 1914, M'Leod (2) reviewed the literature, and although he believed that there was an intimate connection between hemolytic power and virulence, he considered that there had been no solution of the problem which had been generally accepted. To attempt a solution of the problem purely on a clinical basis is obviously impossible on account of the great variations in susceptibility of individuals to infection. This is undoubtedly the reason that observations depending on the course of any infection as an indication of pathogenicity have led to such indefinite and opposed conclusions. If it is possible to establish definite facts in the laboratory under constant environment suitable for the growth of the bacteria, the same methods can probably be applied to the clinical phase of the problem.

The ideal laboratory experiment has been indicated by M'Leod (3). Briefly, it consists in testing the hemolytic power of the streptococcus *in vivo*; there are, however, no trustworthy methods with which this procedure can be accurately carried out. The obvious substitute is to observe the hemolytic titer of virulent strains in the serum of the animal for which they are pathogenic. We attempted to use rabbits in this manner but had great difficulty in obtaining strains which were

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of constant virulence, and, furthermore, it was found that the hemolytic titers of cultures growing in rabbit serum varied considerably. In fact the only medium which gave consistent maximum titers was 20 per cent horse serum broth. Owing to these difficulties we have attempted first to determine the relation between pathogenic and non-pathogenic strains in respect to their lytic power, when they are grown in the serum of an animal for which their virulence has not been especially increased.

Method.

Media.—The character of the media employed is undoubtedly the most important essential for the production of strong hemolysin. Beef infusion broth to which horse serum had been added to 20 per cent of the volume was found to be the most satisfactory for the comparison of various strains. If the broth is made with 2 per cent peptone the titers are quite constant in different experiments with the same streptococcus. To ensure uniformity all the media used in this series were made at the same time from the same lot of beef infusion. They were titrated so that the pH was 7.6 after sterilization, and were distributed in quantities of 80 cc. in 250 cc. Pyrex flasks. The horse serum was obtained from the same horse in each instance. While it was still fresh 20 cc. were added to each flask. The contents of the flasks were then inactivated at 56°C. on 3 successive days and stored on ice until used. In this way it was possible to grow the cultures under conditions in which the available protein substances and the antihemolysins were constant.

Seeding and Bacterial Counts.—The second factor which may vary sufficiently to destroy the accuracy of the results is the nature of the seeding. According to De Kruif and Ireland (4) young cultures produce streptolysin more rapidly than those which have ceased to grow actively. Since it had been previously found (5) that, under the conditions which we employed, the period of active growth ceased after about 16 hours, the flasks were seeded with horse serum broth cultures from 14 to 18 hours old. The seeding was controlled by counting a dilution of bacteria in a weak aqueous solution of methylene blue. A Helber counting chamber with a thin cover-glass was used so that the count could be made with either a high power dry lens

or with an oil immersion lens. The chains of bacteria were counted and the average number of individuals in each group was estimated by counting the cocci in a film made from the dilution pipette. It was possible to approximate the number of cocci in each cubic centimeter of the culture used for seeding by this method. On account of the inaccuracy of the bacterial counts each experiment was done in duplicate or triplicate by seeding several flasks so that they contained approximately 1, 5, and 10 million per cc. The flasks were counted a second time before incubation by the dilution and plating method described in a previous article (5), and the growth of the cultures was followed at intervals in a similar manner. It was impossible to count the streptococci with any great degree of accuracy, but by seeding several flasks with varying numbers of cocci, a comparable growth was obtained in at least two. After several experiments it was found that the counts were unnecessary because in the tests in which the flasks were seeded within a fairly close range, the minimum hemolytic dose was the same in each flask of the triplicate experiments.

Hemolysin Titers.—Hemolysis was determined with the supernatant fluid obtained after centrifuging a portion of the culture at high speed for 10 minutes. The supernatant fluid was set up in a series of tubes with 1 cc. of 5 per cent mouse cells washed and suspended in isotonic salt solution. The smallest quantity of fluid necessary to hemolyze these cells completely without leaving sediment was called the titer of the culture. These determinations were made throughout the experiment, but more frequently during the time that streptolysin production was at a maximum. Since it is impossible to compare titers with different suspensions of red cells, a sufficient quantity was prepared to complete a single experiment. The character of the red cells does not change sufficiently when they are suspended in isotonic salt solution to vitiate results obtained within a period of 16 to 18 hours after the cells are placed in suspension. The titers obtained by these methods were practically constant for each strain.

EXPERIMENTAL.

Five strains of beta type streptococci obtained from acute human infections, in some instances from the blood stream and in others from pleural exudates, were used. They conformed to the *Strepto-*

coccus pyogenes of Holman (6) and gave a final hydrogen ion concentration of pH 4.9 to 5.2 in glucose broth. Before these strains were used for the present experiments they were stored on blood agar and transplanted at frequent intervals during a period of several months, in order that they might lose their original virulence for animals.

At the beginning of the experimental work the invasive power of each strain was determined on mice of approximately the same weight. They were then passed through mice by intraperitoneal injection and obtained in pure culture from the heart's blood. The doses were regulated so that the animals died within 24 hours. After each passage the strain was transferred to rabbit blood agar in the second subculture. When each streptococcus was sufficiently invasive, the avirulent and virulent forms were transplanted from the blood agar tubes into horse serum broth, then, after an interval of 14 to 18 hours, the trial flasks of bouillon were seeded with the necessary quantities of these cultures. In this way the streptolysin production was determined with actively growing cocci which were accustomed to the media in which the test was made. After the hemolysin tests, the virulence of the streptococci was again determined on mice with the corresponding subcultures from the stock media.

Experiment 1.—Strain J, which had been in stock culture for 2 months was passed through twenty mice. At the end of the passages the original culture and the virulent mouse strain were grown in horse serum broth and seeded into 100 cc. flasks of media. The seedings were made so that there were three flasks for each strain containing approximately 1, 5, and 10 million cocci per cc. The bacterial counts (Text-fig. 1) and the hemolysins were determined at frequent intervals until after the maximum streptolysin production had occurred. The pathogenicity for mice was determined immediately after the experiment, with a 24 hour plain broth culture of Strains J(0)5 and J(20)5.[1] The results of the tests are shown in Tables I and II.

Experiments 2 to 5.—The previous experiment was repeated with Strains K, M, R, and S. Only two seedings were used for each strain, however, one of 1 and one of 10 million cocci per cc. Table III summarizes the maximum titers, the hour at which they occurred during the growth of the culture, and the pathogenicity of the various strains for mice.

[1] The strains have been indicated by letters, the number of passages by a figure in parentheses, and the subculture by the second numeral.

TABLE I.

Streptolysin Production by Strain J.

Interval.	1 million.*		5 million.		10 million.	
	Count.	Titer.†	Count.	Titer.	Count.	Titer.
	Strain J(0)5, not passed through animals.					
hrs.		*cc.*		*cc.*		*cc.*
0	1.1 (10^6)‡	0.0	5.5 (10^6)	0.0	1.1 (10^7)	0.0
3½	5.0 (10^7)	0.0	4.7 (10^8)	0.0	6.0 (10^8)	0.6
6		0.02		0.04		0.04
7½	1.0 (10^9)		1.3 (10^9)		1.0 (10^9)	
8		0.06		0.08		0.08
10		0.06		0.2		0.15
12	8.0 (10^8)		7.5 (10^8)		2.0 (10^9)	
	Strain J(20)5, after passage through animals.					
0	2.4 (10^6)	0.0	1.2 (10^7)	0.0	2.4 (10^7)	0.0
4	1.5 (10^7)	0.0	7.3 (10^7)	0.0	5.6 (10^8)	0.0
5½		0.4		0.06		0.08
7		0.4		0.04		0.04
8	6.5 (10^8)		1.6 (10^9)		7.8 (10^8)	
9		0.04		0.06		0.06
11	9.8 (10^8)	0.04	7.0 (10^8)		8.1 (10^8)	
12		0.06				

* The counts designated in millions were calculated from the direct counts used for seeding. The others are expressed in powers of ten.

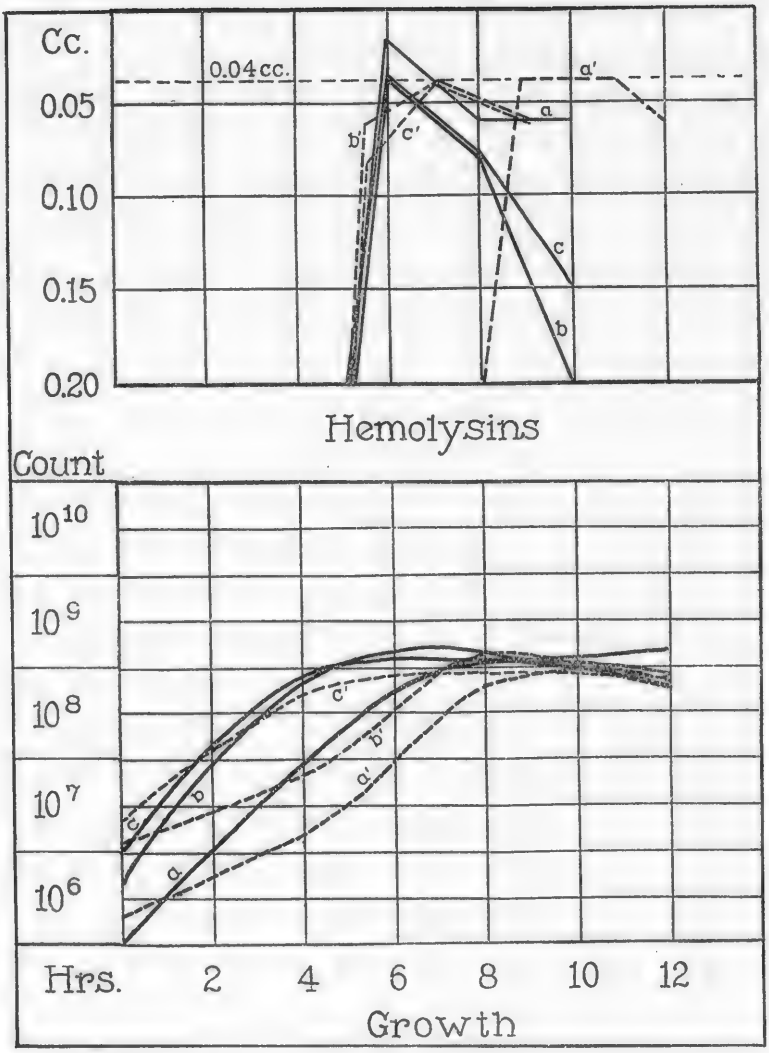
† The titers are the smallest amounts of the supernatant fluid which completely hemolyzed 1 cc. of the cell suspension after 1 hour at 37°C.

‡ All counts in the columns were made from dilutions and plates.

TABLE II.

Pathogenicity of Strain J for Mice.

Strain.	Quantity of culture.	Length of time animal survived.
	cc.	*hrs.*
No. J(0)5, not passed through animals.	1.4	18
	1.2	18
	1.0	(Recovered.)
	0.8	"
	0.6	"
No. J(20)5, after passage through animals.	0.2	17
	0.1	17
	0.08	17
	0.06	18
	0.02	(Recovered.)



----- Strain J (20) 5.
——— Strain J (0) 5.

TEXT-FIG. 1. Comparison of the growth and of hemolysin production by Strain J before and after mouse passage. The counts are charted logarithmically. Curves *a* and *a'* give the results obtained with original seedings of 1 million, Curves *b* and *b'* with original seedings of 5 million, and Curves *c* and *c'* with original seedings of 10 million.

TABLE III.

Streptolysin Production and Pathogenicity for Mice of Strains J, K, M, R, and S.

Strain No.	Seeding.	Titer.	Time at which maximum titer occurred.	Pathogenicity.*
	million†	*cc.*	*hrs.*	*cc.*
J(0)5	1	0.02	6	1.2
	5	0.04	6	
	10	0.04	6	
" (20)5	1	0.04	9	0.06
	5	0.04	7	
	10	0.04	7	
K(0)2	1	0.04	7½	1.0
	10	0.04	7½	
" (17)2	1	0.04	12	0.1
	10	0.04	12	
M(0)4	1	0.04	10	‡
	10	0.02	8	
" (19)4	1	0.02	8	0.5
	10	0.02	8	
R(0)4	1	0.04	8	0.4
	10	0.01	8	
" (19)4	1	0.02	8	0.03
	10	0.04	8	
S(0)2	1	0.04	6½	1.0
	10	0.04	6½	
" (17)2	1	0.04	10	0.2
	10	0.04	8	

* The pathogenicity is indicated by the smallest quantity of a 24 hour broth culture fatal to mice in 24 hours.

† Based on direct counts and controlled by dilution and plating.

‡ Animals survived doses of 5 to 10 cc. of centrifuged culture.

DISCUSSION.

From the results of these experiments it appears that both the virulent and avirulent types of each strain produce approximately the same maximum titer at some time during the growth of the cultures. Grown under the conditions previously described the strains have yielded titers in the majority of instances of 0.04 cc.

It is not to be expected that various experiments can be compared in this respect, when they are carried out on different days with different red cell suspensions. The quantities of culture fluid required to hemolyze the cell suspension in any one experiment are fairly consistent, with the exception of one in which Strain R was used in seeding. A certain degree of variation can be anticipated because of the difficulty experienced in reading the last completely hemolyzed tube in a series graded in 0.01 cc. With two of the five strains, M and R, the peaks of the hemolysin curves were simultaneous in the cultures of both the virulent and less virulent forms. With Strains J, K, and S the former lagged from 1 to $4\frac{1}{2}$ hours. This lag was apparently due to the fact that the more virulent strains grew more slowly during the first hours of incubation. This fact was evident from the amount of cloudiness in the flasks as well as from the plate counts. In the two instances in which the highest points of the hemolysin curves were reached simultaneously, the cultures of the strains which had not been passed through animals were moderately hemolytic for some time before the other flasks had any destructive action on red cells.

CONCLUSION.

Strains of streptococci whose virulence has been increased for any one species of animal do not produce greater concentrations of hemolysin than the original strain. Furthermore, there is a tendency for the original culture to grow more rapidly than the more pathogenic form, and to reach the height of hemolysin production at an earlier stage during the growth of the culture. These conclusions can probably be applied only to experiments in which the serum used in the media is from some species not employed for the animal passages.

BIBLIOGRAPHY.

1. Marmorek, A., *Ann. Inst. Pasteur*, 1902, xvi, 169.
2. M'Leod, J. W., *J. Path. and Bact.*, 1914–15, xix, 392.
3. M'Leod, J. W., *J. Path. and Bact.*, 1911–12, xvi, 321.
4. De Kruif, P. H., and Ireland, P. M., *J. Infect. Dis.*, 1920, xxvi, 285.
5. Stevens, F. A., and Koser, S. A., *J. Exp. Med.*, 1919, xxx, 539.
6. Holman, W. L., *J. Med. Research*, 1916, xxxiv, 377.

QUANTITATIVE DISTRIBUTION OF PARTICULATE MATERIAL (MANGANESE DIOXIDE) ADMINISTERED INTRAVENOUSLY TO THE DOG, RABBIT, GUINEA PIG, RAT, CHICKEN, AND TURTLE.

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(Received for publication, November 11, 1920.)

The convenient method for manganese determination developed by Reiman and Minot (1) has been utilized by Drinker and Shaw (2) in a series of observations upon the distribution of finely divided manganese dioxide introduced intravenously in the cat. A discussion of the extensive literature upon the intravenous injection of insoluble foreign particles, together with a detailed description of the technique employed in their experiments, will be found in the latter paper. Since plans for future work have made necessary the use of other laboratory animals than the cat, and since the possibility of differences between species in the ability to clear the blood stream of foreign material has been insufficiently investigated, a similar study to that made by Drinker and Shaw (2) with the cat was undertaken with other common laboratory animals. The results are reported in the present paper.

EXPERIMENTAL.

The suspensions of manganese dioxide employed contained no particles above 1 micron in size, and on analysis varied in manganese content from 0.140 to 0.903 mg. of manganese per cc. The number of particles varied between 2.5 and 5 billion per mg. of manganese. Tissues to be analyzed were taken from the body immediately after the animal had been bled to death, and the results are expressed in milligrams of manganese per 100 gm. of wet tissue. The results of Bertrand and Medigreceanu (3), with work of Reiman and Minot (1) and with analyses made by ourselves, indicate that the normal

manganese content of the tissues reported in this paper is negligibly small. The amounts shown in Table I are of no significance in view of the comparatively large injections made—large enough to assure detection of manganese in any organs in which manganese dioxide particles lodge.

In all instances injections of manganese dioxide suspensions were made under urethane anesthesia, and in the dog and rabbit records of blood pressure and respiration show, as in the cat (2), that no toxic

TABLE I.

Average Normal Manganese Content per 100 Gm. of Wet Tissue.

Organ.	Amount of manganese per 100 gm. of wet tissue.						
	Fifteen cats.	Eight dogs.	Eight rabbits.	Two guinea pigs.	Two rats.	Two chickens.	Two turtles.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Liver	0.341	0.238	0.178	0.371	0.326	0.311	0.119
Spleen	0.069	0.022	0.054				
Brain	0.035	0.044	0.062				
Stomach	0.046	0.043	0.059				
Small intestine	0.044	0.028	0.068	0.137*	0.099*	0.051*	0.061*
Colon	0.086	0.080	0.099				
Cecum and appendix			0.043				
Kidney	0.136	0.087	0.147	0.000	0.110	0.124	
Lung	0.031	0.023	0.036	0 000	0.000	0.000	0.000
Muscle	0.025	0.020	0.022	0.043	0.000	0.030	0.016
Bone			0.101				
Urine	0.000	0.000	0.000				
Blood	0.000	0.000	0.000				

* Entire intestinal tract.

symptoms attend such injections. While records were omitted during the injection of the smaller animals no unfavorable symptoms were noted.

The injected animals were killed by bleeding to death from the carotid arteries at the end of 1 hour. Organs for analysis were at once removed, weighed wet, and the manganese content was recorded in milligrams per 100 gm. of tissue. Drinker and Shaw (2) found for the cat that the liver, lungs, and spleen are the points of maximum lodgment, and our observations indicate that this is also true for

other animals. Table II shows the small amounts removed by tissues exclusive of the liver, lungs, and spleen in the rabbit. Table III presents the percentages of injected manganese deposited in the liver, lungs, and spleen of the animals employed. A column is also included, which obviously cannot be completely accurate, to represent the traces found in the blood, and a final column represents the percentage of injected material deposited in other organs than those

TABLE II.

Distribution of Manganese in Tissues Exclusive of the Liver, Lungs, and Spleen in the Rabbit after the Intravenous Injection of Manganese Dioxide.

Weight 2.6 kilos. Mar. 9, 1920. Injection of 13.6 cc. of manganese dioxide suspension containing 19.3 mg. of manganese per 100 cc. Total injection 2.62 mg. of manganese.

Organ.	Amount of manganese recovered.	Per cent of injected manganese recovered.
	mg.	
Brain	0.000	0.00
Stomach	0.000	0.00
Small intestine	0.031	1.2
Colon and rectum	0.013	0.5
Cecum and appendix	0.000	0.00
Kidney	0.007	0.3
Bone	0.004*	2.2†
Heart	0.002	0.1
Blood	0.022	0.7‡
Total	0.079	5.0

* Tibia.

† Bone weight of body calculated at 5 per cent of body weight.

‡ Blood weight of body calculated at 7 per cent of body weight.

chiefly concerned in removing foreign particles from the blood stream. The latter amount varies between 19 per cent in one guinea pig and 2 per cent in one rat. It is impossible to foretell why such variations occur and where the manganese will be found. In the rabbit even during the 1st hour following injection there is a variable degree of excretion in the bile. Some of this manganese is thus included in analyses of the duodenum and jejunum. The intestinal wall of all

the animals studied shows small and very variable amounts of deposition. The same is true of muscle and kidney.

Text-fig. 1 is a graphic representation of the data from Table III. The most striking feature of the figure is that the lungs of the cats contain practically 50 per cent of the manganese 1 hour after injection, while the lungs of all the other animals contain minute amounts, Dog 1 with 14 per cent being the single exception to this.

TABLE III.

Distribution of Manganese 1 Hour after the Intravenous Injection of Manganese Dioxide.

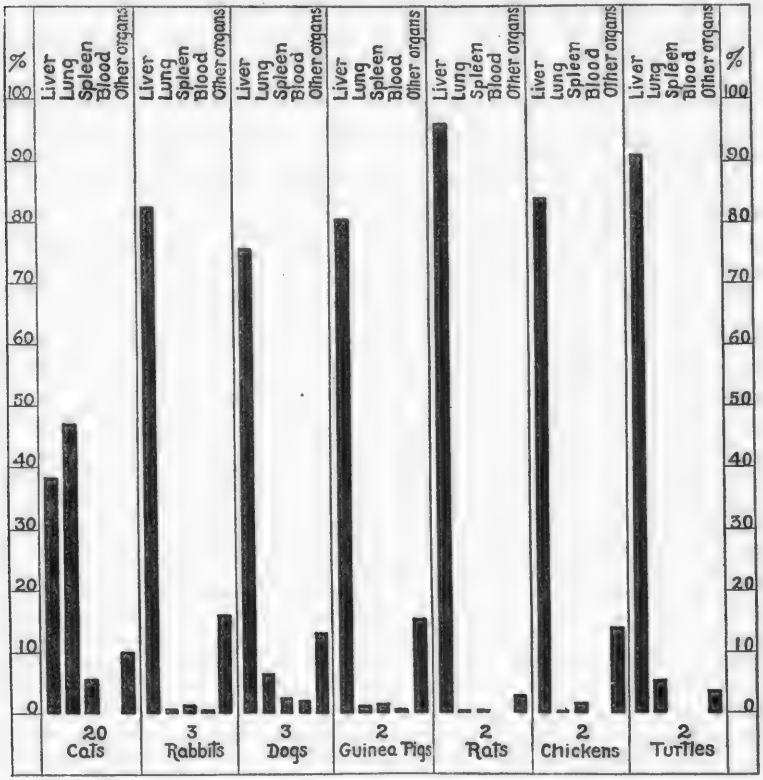
Animal.	Amount of manganese injected.	Per cent of injected manganese recovered.				
		In liver.	In lungs.	In spleen.	In blood.*	In other organs.
	mg.					
Average for twenty cats		38.3	47.0	4.7	0.0	10.0
Rabbit 1	7.98	81.0	1.2	0.8	0.6	16.5
" 2	6.18	81.0	0.2	2.5	0.7	15.4
" 3	2.71	85.6	0.52	0.55	0.0	13.3
Dog 1	83.5	64.0	14.0	2.0	3.9	16.1
" 2	45.2	83.0	3.7	2.1	2.1	9.1
" 3	67.7	79.0	2.0	4.0	0.9	14.1
Guinea Pig 1	2.03	76.0	2.1	1.7	1.2	19.0
" " 2	2.34	85.0	0.8	1.4	0.3	12.5
Rat 1	1.34	94.0	0.5	1.9	0.0	3.6
" 2	0.83	98.0	0.6	0.0	0.0	2.0
Chicken 1	4.36	84.0	0.0	1.7	0.0	14.3
" 2	2.82	84.0	0.4	1.7	0.0	13.9
Turtle 1	1.13	94.0	3.0	No analysis.	0.0	3.0
" 2	1.0	88.0	7.5	" "	0.0	4.5

* Blood weight calculated at 7 per cent of body weight.

In the animals whose lungs removed less of the suspension from the circulating blood the liver removed more manganese so that the amount scattered among the other tissues was very little greater or was even less than in the cats.

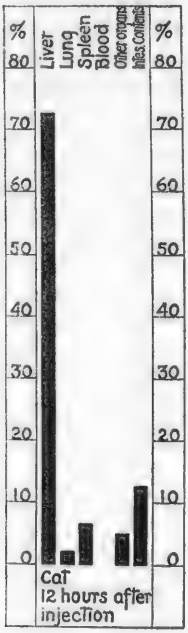
In Text-fig. 2 the distribution of manganese dioxide 12 hours after injection in the cat is shown. At this period the animal had lost the high lung concentration which was observed 1 hour after injection. The material had accumulated in the liver, from which it slowly

escaped through the bile. With the rabbit and guinea pig killed 15 minutes after injection, no difference was found in distribution of manganese dioxide from that shown in Text-fig. 1. These animals,



TEXT-FIG. 1. Distribution of manganese in the organs of animals 1 hour after the intravenous injection of manganese dioxide.

therefore, unlike the cat do not go through the stage of deposition in the lungs and final collection in the liver, but the foreign material is at once localized where its removal from the body is best accomplished.



TEXT-FIG. 2. Distribution of manganese in the organs of a cat 12 hours after the intravenous injection of manganese dioxide.

DISCUSSION AND CONCLUSIONS.

The experiments presented may be contrasted with but little other work with injected particles, since previous experimentation has lacked any quantitative basis. Voigt (4) has published a series of papers on the distribution of colloidal silver and colloidal silver iodide. He determined the distribution both chemically and microscopically. His suspensions differed from ours in several ways. In the first place, the individual particles of his suspensions were much finer, having an average diameter of 15 to 20 micromicrons in contrast to the 0.6 to 1 micron diameter of the manganese dioxide particles used in the present study. Also, the concentration of Voigt's suspensions was far greater, ranging from 10 to 50 per cent in contrast to 0.015 to 0.09 per cent in our work. Furthermore, some of his suspensions must have shown agglutination, since he found emboli in the lungs of rabbits. In spite of these differences, three rabbits, killed at a longer interval following injection than the animals in our experiments, displayed 0.2, 5.1, and 3.7 per cent of the injected material in the lungs, out of a total of 38, 89, and 63 per cent recovered on analysis of all tissues.

In the same experiments the livers contained respectively 35, 80.7, and 51.8 per cent of the material injected. These rather rough results agree with our own and confirm the indication that in the rabbit the lungs play a very minor part in clearing the blood of foreign particles.

More recently Duhamel (5) has published a brief note upon injections into rabbits of colloidal platinum, selenium, mercury, copper, and iron. On chemical analysis he found a large proportion of the injected material in the liver. Again the individual particles were very small but the function of the liver in removing them from the circulation is obvious.

It has been recognized for many years that soluble salts of the heavy metals are deposited from the blood in the liver, and that this deposition plays an important part in the removal of such salts from the body. Clearly, the organ operates with an equal degree of efficacy in the case of particles up to 1 micron in diameter, and probably would do so with even larger sizes, though as yet we have no data upon the subject.

As Drinker and Shaw (2) have discussed much of the bacteriological work relating to removal of organisms from the blood stream, it is only necessary here to mention such data as display differences between the lung element in the reaction. Bartlett and Ozaki (6), using histological methods for detection, found larger numbers of staphylococci in the lungs of dogs and rabbits killed 1 to 30 minutes after injection than in other organs. By the end of an hour fewer bacteria were detected in the lungs than in the liver and spleen. Arima (7) found few bacteria (*Staphylococcus aureus*, *B. coli*, and *B. typhosus*) in the lungs of rabbits 1 hour after injection. Kyes (8) found few pneumococci in the lungs of pigeons. These observations, with the exception of those of Bartlett and Ozaki (6), agree well with our findings in the case of manganese dioxide particles. It is only possible to express a rough opinion as to quantities of material on the basis of histological examination, and we are thus inclined to believe that the atypical results of Bartlett and Ozaki (6) represent an error in judgment and do not affect the facts of the case.

The most important and conclusive work with bacteria is that of Hopkins and Parker (9). They found a marked difference in distribution of streptococci injected intravenously into cats and rabbits. Using comparatively accurate cultural methods they found the following distribution of streptococci 10 minutes after injection.

The result is in conformity with that obtained in the present study for manganese dioxide particles of approximately the same size as streptococci. It is not known why the cat and the rabbit display these differences, but fortunately the cat is unique among the labo-

Organ.	No. of streptococci per 0.1 gm. of tissue.	
	In cat.	In rabbit.
Lung	315,000	41,000
Liver	34,000	104,000
Spleen	18,000	120,000
Kidney	100	0
Bone marrow	500	
Psoas	8	1,500
Pectoralis	27	
Blood	26	

ratory animals tested and is seldom used for bacteriological work.

SUMMARY.

The distribution of manganese dioxide particles 1 hour following intravenous injection in cats, dogs, rabbits, guinea pigs, rats, chickens, and turtles is described. This distribution is remarkably constant for all the animals tested, except the cat, in which the injected material is practically equally divided between the lungs and liver. In the other animals the liver performs the main share of the work, and in the cat it has been shown that the liver after 12 hours accumulates the manganese which was formerly deposited in the lungs.

The results are in harmony with experiments in which bacterial suspensions are employed for injection and confirm the suggestion previously made (2) that in the first handling of foreign particulate material the animal behaves similarly whether protein or inorganic injections are used.

BIBLIOGRAPHY.

1. Reiman, C. K., and Minot, A. S., *J. Biol. Chem.*, 1920, xlii, 329.
2. Drinker, C. K., and Shaw, L. A., *J. Exp. Med.*, 1921, xxxiii, 77.
3. Bertrand, G., and Medigreceanu, F., *Ann. Inst. Pasteur*, 1913, xxvii, 282.
4. Voigt, J., *Biochem. Z.*, 1914, lxii, 280; lxiii, 409; 1915, lxviii, 477; 1918, lxxxix, 220.
5. Duhamel, B.-G., *Compt. rend. Soc. biol.*, 1919, lxxxii, 724.
6. Bartlett, C. J., and Ozaki, Y., *J. Med. Research*, 1916–17, xxxv, 465.
7. Arima, R., *Arch. Hyg.*, 1911, lxxiii, 265.
8. Kyes, P., *J. Infect. Dis.*, 1916, xviii, 277.
9. Hopkins, J. G., and Parker, J. T., *J. Exp. Med.*, 1918, xxvii, 1.

EXPERIMENTAL STUDIES ON YELLOW FEVER IN NORTHERN PERU.

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(Received for publication, August 7, 1920.)

Leptospira icteroides, first isolated from certain cases of yellow fever in Guayaquil,[1] and later from a case of this disease in Merida,[2] had assumed such significance in the study of the etiology of yellow fever as to make further investigations advisable. Peru, which has had many visitations of yellow fever, was again invaded in June, 1919, in the province of Piura, the northernmost region, bordering on Ecuador. From this invasion a small epidemic arose which had not entirely disappeared in May, 1920. During the outbreak the following towns were affected: Sechura, Morropon, Tambogrande, Chulucanas, Piura (500 cases among the 10,000 inhabitants) in 1919, and Payta (108 cases among 3,000 inhabitants) in 1920. The mortality was estimated to have been about 10 per cent, which is considerably lower than was the case with yellow fever in Guayaquil and Merida, where it was about 50 per cent. An expedition to Peru was therefore undertaken.[3] The present report deals with the results of bacteriological studies at Payta, Piura, and Morropon extending over a period of 3 months, March, April, and May, 1920.

When one of us reached Peru (March 1, 1920) Payta was the only town where the epidemic of yellow fever was still in progress; the last case occurred there on April 16. The first experiments were

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159.

[2] Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxii, 601, 627.

[3] This expedition was undertaken under the auspices of the International Health Board of The Rockefeller Foundation, and The Rockefeller Institute for Medical Research. We wish to thank the federal and local authorities in Peru for their courtesy and cooperation in this work.

carried out in Payta. In April an epidemic was reported at an inland town, Morropon, and a trip was made to that place to secure material for further studies to be carried out in Piura, where better laboratory facilities were available.

Studies in Payta.

Payta, a town of about 3,000 inhabitants, is the principal port in northern Peru. It consists of a cluster of bamboo huts on a strip of sandy shore. Rain is rare, and water is very scarce. The water supply comes from a river about 7 kilometers from the town, but the amount is hardly sufficient for ordinary daily needs.

A provisional laboratory was set up in Payta in a small bungalow consisting of three rooms. One room served as a laboratory, another as an animal room, and the third as a sleeping room. The laboratory supplies brought from The Rockefeller Institute had been resterilized in the laboratory of the Municipal Institute of Hygiene in Lima, as there were no facilities for steam or hot air sterilization at Payta. The rabbit serum used in the culture media was brought from New York and as a result of the long voyage in a tropical climate a precipitate had appeared in it. The guinea pigs, also brought from New York, had suffered severely, and about two-thirds of them had succumbed within 2 weeks of their arrival in Payta. The feed for the guinea pigs was scarce, so that only the larger and hardier animals survived. This was unfortunate, as the larger animals are less suitable for initial inoculation; however, a certain number of native guinea pigs was procured. A very serious circumstance was the fact that, owing to the lack of electric current, the dark-field microscope could not be used. Moreover, the effort to obtain Giemsa preparations of the blood were unsuccessful because of the quality of the water. Since most yellow fever patients were treated by their physicians at their own homes it was not always easy to secure consent to obtain blood for inoculation, and it was practically impossible to obtain blood twice from the same patient. Finally, the cultures of *Leptospira icteroides* brought to Payta from Merida did not survive the journey.

Under the adverse conditions and the lack of laboratory facilities, the bacteriological work was confined to cultivation and animal

transmission with such samples of blood as could be obtained, the object being to infect guinea pigs and to produce the characteristic symptoms and lesions in these animals. In all, nine cases of yellow fever were studied. Guinea pigs brought from New York were inoculated from seven and native guinea pigs from two of the cases.

Case 1 (*Fatal*).—B. Onset Mar. 10, 1920. Mar. 12. Temperature 39°C.; pulse 110; albuminuria. Mar. 14. Temperature 38.5°; pulse 88; albumin 1 gm. per 1,000 cc. Mar. 16. Delirious; black vomit; anuria. Mar. 17. Died.

Mar. 12 (3rd day of illness). Blood taken and cultures made (nine tubes). Mar. 16. 1 cc. of citrated blood (kept in the ice box for 4 days) was inoculated intraperitoneally into Guinea Pig 1 and 2 cc. into Guinea Pig 2. The culture tubes, which had stood at room temperature for 4 days, appeared free from contamination, and three guinea pigs were inoculated with material from Tubes 1 to 4 and three with material from Tubes 5 to 9. Some of the culture material was left standing until Mar. 23, when it was inoculated into three guinea pigs.

The two guinea pigs inoculated with blood 4 days old showed no fever. A few old hemorrhagic areas were found in the lungs of Guinea Pig 2 when it was killed on the 15th day. Of six guinea pigs inoculated with culture material 4 days old, three had fever on the 6th day and showed petechial lung hemorrhages when killed on the 15th day. The three remaining animals either suffered from an intercurrent infection (pneumonia) or escaped any obvious infection.

Of the three guinea pigs inoculated with the 11 day culture material, one (Guinea Pig 43) showed petechial hemorrhages in the lungs when killed on the 15th day. The other two showed on autopsy no leptospira lesions but enlarged spleen and pulmonary congestion, which were taken as evidence of secondary infection.

The findings described show that in no instance was a fatal infection by *Leptospira icteroides* induced, but they raise the question whether the characteristic hemorrhagic areas in the lungs in Guinea Pigs 2, 3, 5, 8, and 43 did not indicate a mild infection with this organism.

Case 2 (*Moderate*; *Recovered*).—C. Onset Mar. 13, 1920. Moderate case; course typical. Mar. 20. Recovered.

Mar. 14 (2nd day of illness). Blood taken and used wholly for making cultures. Three guinea pigs were injected with a 3 day culture, three with a 4 day culture, and three with a 9 day culture.

Of this series almost all, except Guinea Pig 14, which received a 3 day culture, and No. 19, which received a 4 day culture, died of intercurrent infections (pneumonia, paratyphoid, cocci), while two remained well. Guinea Pig 14 showed on the 5th day a temperature of 39.7°C., and on the 6th 39.6°, while at autopsy the lungs showed several hemorrhagic areas. Guinea Pig 19 showed on

the 7th day a temperature of 39.5° and on the 8th and 9th days 39.8°. It was killed on the 10th day for examination and transfers. Few small petechiæ in the lungs and minute points of hemorrhage in the right kidney. The blood and organ emulsions from this animal were inoculated on Mar. 28 into two guinea pigs, both of which soon returned to normal.

The experiments on Case 2 are suggestive and lead to the tentative conclusion that in two at least of the nine guinea pigs inoculated with culture materials prepared with blood drawn on the 2nd day a mild infection with *Leptospira icteroides* was induced. It is possible that Guinea Pig 19, if allowed to live longer, might have developed a typical form of the *icteroides* infection, as, when killed on the 10th day, definite lesions were present in the lungs and kidney. The failure to transfer the infection from this animal into two others is not conclusive, as in the early transfers a larger number of guinea pigs should be employed, because of the resistance to infection which certain guinea pigs usually exhibit.

Case 3 (recovered) gave similar results with blood drawn on the 3rd day of illness.

Two guinea pigs were inoculated with 2 cc. of blood from Case 4 (fatal; blood drawn on 2nd day of illness) soon after it was drawn, and two with the same amount of blood from Case 5 (recovered; blood drawn on 3rd day of illness). Cultures made with blood from each of these patients were left at room temperature for 4, 7, and 10 days and then inoculated into eight guinea pigs (Mar. 19, 22, and 26). Some of the animals inoculated with material from Cases 4 and 5 had definite febrile reactions and showed at autopsy lung lesions suggestive of a mild leptospira infection, but there was no fatal infection with typical jaundice.

With Case 6 (recovered; blood drawn on Mar. 19, 2nd day of disease) and Case 7 (recovered; blood drawn on Mar. 20, 3rd day of disease), the blood was drawn into citrate serum agar mixed in equal parts, and 3 cc. of the mixture were inoculated into each of two guinea pigs. Cultures made in the usual way were allowed to stand at room temperature for 3 to 7 days, and two sets of six guinea pigs were inoculated with this material. The results with Cases 6 and 7, both with blood and cultures, were unsatisfactory. The majority of the guinea pigs showed irregular febrile reactions, and from these animals, owing to the scarcity of guinea pigs, no transfers were made. Some of them, when killed later, were found to have hemorrhagic areas in the lungs, some showed indications of secondary infection (enlarged spleen), while others showed no lesions. In no instance was there a typical fatal leptospira infection.

Two more cases were studied before the epidemic in Payta subsided, Case 8 (recovered; blood drawn on 3rd day of illness, Mar. 29) and Case 9 (recovered;

blood drawn on Apr. 1, 2nd day of illness). In view of the failure to secure a definite transmission with fatal outcome with the larger guinea pigs still alive from the lot brought from New York, we decided to test native guinea pigs. For this purpose six native and two American guinea pigs, weighing 600 gm., were inoculated with a 7 day culture of the blood of Case 8 and five native guinea pigs with the 10 day culture from Case 9. None of the animals developed a typical fatal leptospira infection, although some undoubtedly had a mild infection, since lung lesions were found at autopsy and in two instances there was a suggestion of jaundice. It is interesting to note here that later experiments demonstrated that the native guinea pigs possess a greater resistance to the *icteroides* infection than the domesticated variety brought from the United States.

It is obvious that in the transmission experiments just described as having been carried out at Payta, no typical instance of fatal infection with *Leptospira icteroides* was obtained, and in no instance was the leptospira observed under the microscope. As the dark-field microscope was not available and no proper Giemsa staining could be secured, the latter circumstance is without value.

On the other hand, certain positive results were obtained in inoculated guinea pigs which led to the belief that a mild form of *Leptospira icteroides* infection had in some instances been induced; *i.e.*, rise of temperature after the period of incubation common in this infection (3 to 5 days) and at autopsy definite hemorrhagic areas in the lungs and in one instance in both lungs and kidney, with occasionally a suggestion of jaundice. The failures to obtain more pronounced results are not difficult to account for. As stated above, almost all the guinea pigs of the most favorable age and weight shipped from New York succumbed *en route* or soon after arriving at Payta. Those remaining were so few in number that they were used sparingly; hence fewer were injected with given samples of blood or cultures than would ordinarily have been employed. The rabbit serum which is essential to successful cultivation of the leptospira had undergone changes with the formation of a precipitate, and the reaction became so alkaline as to prevent a growth of *Leptospira icteroides* to any extent. And yet a degree of success, which was confirmed by subsequent results, was, we believe, achieved.

Studies in Piura.

The epidemic having subsided in Payta, the laboratory was removed to Piura, where sterilizing facilities and adequate water were available. A laboratory was set up, through the cooperation of the government, in the Belan Hospital. A detached building was used for animal quarters and feed was also more plentiful.

By the time the small laboratory had been started cases of yellow fever were reported in Morropon, a small town of 2,000 inhabitants in the foothills of the Andes. The distance from Piura was about 65 miles, a desert separating the two places. Arrangements were made at once to investigate the cases.[4] As the journey was made on horseback it was obviously out of the question to transport experimental animals, etc., by this means through a tropical region; hence it was decided to rely entirely on cultures. Fresh rabbit serum was obtained from local rabbits, and in order to guard against adverse changes in the culture media the component parts, consisting of serum, and 0.3 per cent semisolid agar, were carried separately.

The cultures were made by drawing the blood from an arm vein of the patients directly into the tube of semisolid agar, the rabbit serum then being added in a proportion of 1:5. The whole was thoroughly mixed and covered with a layer of liquid paraffin, and the tubes were carefully capped with tin-foil and carried back to Piura.

On arrival at Morropon it was ascertained that cases of yellow fever had been occurring for some time, and the epidemic was regarded as declining; however, by making house to house visits several cases diagnosed as yellow fever by Dr. Caballero were found on April 23. Between April 24 and 27 cultures with the blood were made from six cases, one of which proved later not to have been yellow fever. The remaining five cases pursued a clinical course which left no doubt as to their yellow fever nature. Because of the illness of one member of the party, the work at Morropon was suspended on April 28.

[4] The journey on horseback from Piura to Morropon usually takes 1½ days. Our party consisted of Dr. Enrico Caballero, the government expert stationed at Catacaos, who showed us every courtesy, Mr. John Mitchell, a sanitary engineer, and Dr. Kligler. The expedition started from Piura on April 21 and arrived at its destination on April 22.

The party returned with the cultures to Piura, reaching there on May 3. At the same time the stock of guinea pigs had been renewed, 300 young, healthy animals having been brought from The Rockefeller Institute. Moreover, because of the lack of electric lighting facilities which had made it impossible to use the dark-field microscope, a storage battery suited to that instrument had also been brought from New York.

Very few of the tubes showed contamination, the blood still appearing bright red in the upper zone of the media. The cultures were inoculated into guinea pigs on May 6, or 9 to 12 days after they had been set up in Morropon. The inoculation procedure was identical with that employed in Merida[2], the upper portions of several selected tubes of culture from a case being pooled and the mixture inoculated intraperitoneally into six young normal guinea pigs.

Dark-field examination of the culture tubes undertaken the next day (May 7, 10 to 13 days after they were made) revealed the presence of active leptospiras in the cultures from three of the five cases. They were few in number and required careful search in some instances. In some tubes no leptospira was detected. As the details of the experiments show, the inoculation of cultures from four of the five cases induced typical fatal infections in some animals, other animals showing only a mild infection or escaping infection altogether.

Case 10 (*Severe*; *Recovered*).—C. M., male, age 16 years; born in Salitral; resident of Morropon. Onset Apr. 22, 1920, 7 p.m. Headache; backache; pains in muscles; nausea; no vomiting. Apr. 23. Temperature 39.6°C.; pulse 106. Apr. 24. Temperature 39.9°; pulse 100; albumin +. Apr. 25. Temperature 39°; pulse 90; albumin ++; nausea. Apr. 26. Temperature 37.4°; pulse 90; albumin ++; black vomit. Apr. 27. Temperature 37.6°; pulse 78; albumin ++; epigastric pain; epistaxis; urine increasing towards normal amount; jaundice. Apr. 28. Temperature 37°; pulse 76; recovering.

Blood was taken on the 2nd day of illness at 11 a.m. Cultures examined after 12 days contained living leptospiras. Six guinea pigs (Nos. 13 to 18) were inoculated with material from Tubes 1 and 2 on May 6, with positive transmission in all.

Guinea Pig 13.—Temperature 39.2° on the 5th day. Died on the 6th day.

Autopsy.—Epistaxis; subcutaneous petechiæ; marked hemorrhages in lungs and gastric mucosa; jaundice slight.

The emulsions of kidney and liver were inoculated into three guinea pigs (Nos. 39 (Chart 1), 40, and 41), all of which developed typical fatal infection, dying

7, 14, and 15 days after inoculation. The leptospiras were found and successful subcultures made.

Guinea Pig 14.—Killed, when moribund, to obtain infective material for therapeutic experiments to be described in the following paper.[5]

Autopsy.—Numerous petechiæ in lungs; hemorrhage and blood in stomach; liver slightly degenerated; kidneys congested; spleen normal.

Guinea Pig 15.—Killed for transfer 4 days after inoculation, at first rise of temperature to 39.4°C.

Autopsy.—No lesions were noted, but all three of the guinea pigs (Nos. 36, 37, and 38) inoculated with blood and emulsions of liver and kidneys succumbed with typical infection.

Guinea Pigs 16 and 18.—Developed characteristic infection. When jaundice appeared they were utilized for testing the curative effect of the anti-*icteroides* immune serum brought from The Rockefeller Institute, as will be described in the following paper.[5]

With this case leptospiras were found in the initial culture, with which a typical infection was induced in guinea pigs, and further transfer from animal to animal was accomplished. Pure cultures of the leptospira were in turn recovered from the infected guinea pigs.

Case 11 (*Mild*; *Recovered*).—O. V., male, age 18 years; native of Morropon. Onset in afternoon of Apr. 21, 1920. Chills; headache; backache; fever. First seen morning of Apr. 24. Temperature 40°C.; pulse 100; albumin +. Apr. 25. Temperature 38.5°; pulse 70; albumin ++; nausea, but no vomiting. Apr. 26. Temperature 38.5°; pulse 66; albumin ++. Apr. 27. Temperature 37.2°; pulse 58; albumin +; abundant urine. Apr. 29. Temperature 36.6°; pulse 50; mild jaundice; recovering.

Apr. 24 (3rd day of illness). Blood was drawn. The dark-field examination of cultures 13 days after they were made failed to reveal any leptospira, owing to accidental contamination of the tubes while they were being handled the previous day for animal inoculation.

May 6. Six guinea pigs (Nos. 25 to 30) inoculated with culture, then 12 days old. Three of these (Nos. 25, 26 (Chart 2), and 29) developed typical severe infections, while the remaining three showed no perceptible symptoms. When killed for examination, however, all showed some hemorrhagic areas in the lungs, indications of a mild infection. It is interesting to note the different results with the same culture material, due to variations in individual susceptibility of the guinea pig to *Leptospira icteroides*. The symptoms and lesions in fatally infected animals were altogether typical and hence will not be described in detail except in unusual instances. Leptospiras were found in the organ emulsions and a culture was obtained from the blood.

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[5] Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 253.

Transfer from one of these animals was made into three guinea pigs, all of which succumbed in due time to typical fatal infection.

One of the guinea pigs (No. 25) was used, when near collapse, for testing the efficacy of the anti-*icteroides* serum. The animal recovered, having received 1 cc. of the serum.

Case 12 (*Fatal*).—P. C., male, age 28 years; native of mountainous region. Onset, Apr. 23, 1920, typical. Apr. 25. Seen for the first time; temperature 39.4°C.; pulse 102; albumin ++; epigastric pain; no vomit. Apr. 26. Temperature 34.8°; patient in state of collapse; bleeding from nose and gums; black vomit; jaundice. Apr. 27, 6 a.m. Died.

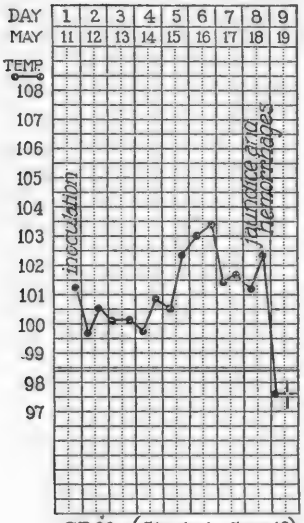
Blood was taken in the morning of the 3rd day of illness. Cultures contained living leptospiras when examined on May 7 (12 days old).

May 6. Six guinea pigs (Nos. 1 to 6) were inoculated with culture material. Of these, three (Nos. 2, 3, and 4) developed severe infections, one dying on the 7th, and one on the 8th day (Chart 3), and the third being killed for transfer on the 6th day, when it was intensely icteric. Three guinea pigs inoculated with blood and liver and kidney emulsions from this animal died with typical symptoms. Three of the six original guinea pigs showed no sign of infection (Nos. 1, 5, and 6), but examination after 12 days revealed hemorrhagic areas in the lungs and also, in one instance, in the suprarenal. Leptospiras were found in the blood and organs in some of the guinea pigs, and cultures were obtained from the blood. The outstanding feature of this strain was the predominance of jaundice which it produced in the animals.

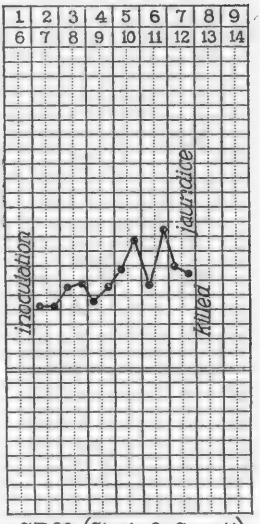
Case 13 (*Moderate*; *Recovered*).—J. C., male, age 14 years; native of Morropon. Onset Apr. 23, 1920. Apr. 26. Seen for the first time; epistaxis; black vomit; melena; temperature 37°C.; pulse 80; albumin ++. Apr. 27. Temperature 36.6°; pulse 66; albumin ++. Apr. 28. Temperature 37°; pulse 80; albumin ++. Recovery.

Apr. 26 (4th day). Blood taken for cultures. Dark-field examination of cultures 11 days after they were made failed to reveal any leptospira. Six guinea pigs inoculated with the 10 day culture material from this case also yielded negative results.

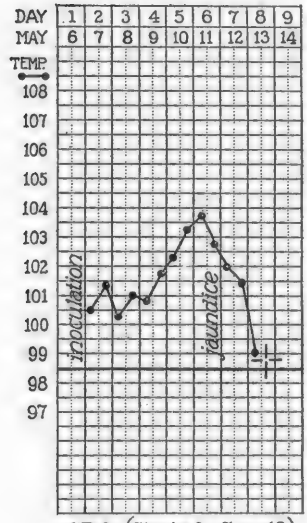
Case 14 (*Severe*; *Recovered*).—F. N., female, age 25 years; native of Morropon. Apr. 25, 1920, 11 a.m. Onset. Apr. 26. Temperature 38.5°C.; pulse 114; albumin trace; headache; backache; muscular pain; face flushed; conjunctivæ congested. Apr. 27. Temperature in morning 39.2°; pulse 106; albumin +. 4 p.m. Temperature 39.5°; pulse 120; severe pain and weakness; nausea, but no vomiting. At request of patient 20 cc. of anti-*icteroides* serum brought from The Rockefeller Institute were injected intravenously. 7 p.m. Temperature 38.9°; pulse 100; stronger; pains relieved. Apr. 28. Afternoon temperature 38.2°; pulse 100; albumin ++; nausea; bilious vomit. Apr. 29. Temperature 38.2°; pulse 90; no nausea; albumin ++. Apr. 30. Temperature 37.9°; pulse 86; mild pharyngitis. May 1. Temperature 36.8°; pulse 80; albumin +; recovering.



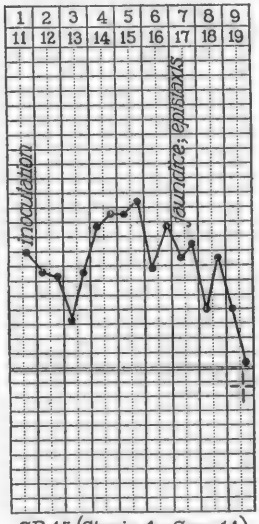
G.P. 39 (Strain 1–Case 10).
Chart 1.



G.P. 26 (Strain 2–Case 11).
Chart 2.



G.P. 2 (Strain 3–Case 12).
Chart 3.



G.P. 45 (Strain 4–Case 14).
Chart 4.

CHARTS 1 to 4. Temperature curves of guinea pigs inoculated with material from yellow fever cases in Peru.

Apr. 27 (48 hours after onset). Blood taken in the morning. The cultures were examined on May 8 when 11 days old, and living leptospiras were found in one tube, apparently dead ones in another.

May 6. Six guinea pigs (Nos. 7 to 12) were inoculated with the 9 day culture material. A typical infection was induced in three, while the other three remained apparently well. One of the latter showed hemorrhagic areas in the lungs when examined after 12 days. The other two had no macroscopic lesions, indicating that they were both completely refractory to this strain.

Transfer was made from one of the positive animals into three guinea pigs, all of which died with typical infections. Leptospiras were demonstrated in varying number in the blood as well as in the emulsions of liver and kidneys, and pure cultures were obtained from the blood of these animals. Chart 4 shows the temperature curve of one of the guinea pigs (No. 45) infected with Strain 4.

In Case 14, as in Cases 10, 11, and 12, positive transmission to guinea pigs was obtained by means of culture material. The initial cultures usually contained living leptospiras. In the blood and liver or kidneys of the infected animals the leptospiras were demonstrated, and a pure culture of *Leptospira icteroides* was recovered from the blood.

Identification of the Morropon Strains.

Upon our return to The Rockefeller Institute, we proceeded with the identification of the strains of leptospira isolated from yellow fever cases in Morropon along the lines previously followed. The Pfeiffer phenomenon was determined, as well as the effects of immune serums upon the organism *in vitro*.

Rich cultures of leptospira strains (Nos. 1 and 2) were employed in these experiments. The serums used were monovalent immune serums prepared in rabbits with Guayaquil Strain 1 of *Leptospira icteroides*. For purposes of control American Strain 2 of *Leptospira icterohæmorrhagiæ* was tested simultaneously. Moreover, a polyvalent immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides* was also tested. The results obtained are given in Table I.

As Table I shows, the leptospira strains from Morropon gave positive Pfeiffer reactions with the immune serums prepared with the Guayaquil strains, but negative reactions with the anti-*icterohæmorrhagiæ* serum. Likewise, with respect to their behavior towards

these serums *in vitro* an indubitable specificity for the anti-*icteroides* serum is evident. The slight reaction with the anti-*icterohæmorrhagiæ* serum may be regarded as a group reaction among closely allied species. It is concluded, therefore, that the leptospiras isolated from Morropon and Guayaquil cases of yellow fever are of the same species.

TABLE I.

Identification of the Morropon Strains.

Mode of test.	Strain No.	Anti-*icteroides* serum (monovalent, rabbit).	Anti-*icteroides* serum (polyvalent, horse).	Anti-*icterohæmorrhagiæ* serum (monovalent, rabbit).	Controls without immune serum.
Pfeiffer (30 min.).	1	Complete disintegration (positive).	Complete disintegration (positive).	For the most part active (negative).	Very active (negative).
	2	Almost complete disintegration (positive).	Complete disintegration (positive).	For the most part active; few appear distorted (negative).	Very active (negative).
In vitro; allowed to stand for 18 hrs.	1	Complete agglutination; some motile leptospiras.	Complete agglutination and immobilization.	Slight agglutination and few immobilized.	Very active; no agglutination.
	2	Complete agglutination; some motile leptospiras.	Complete agglutination; few motile leptospiras.	Partial agglutination and immobilization.	Active; no agglutination.

DISCUSSION AND SUMMARY.

Fourteen typical cases of yellow fever were studied in northern Peru during an epidemic occurring in 1920, nine in Payta in March and April, and five in Morropon and Piura in April and May. The method of investigation was similar to that previously employed, but as the laboratory facilities were very meager certain changes were required. Although in Payta the work was handicapped by the lack of electric light, the scarcity of water and animal food, the unsuitability of the guinea pigs for inoculation, and the changes in culture media due to age, the results obtained under these adverse conditions were by no means negative. While in no instance was there a typical

infection produced in animals, either by direct inoculation of blood or with culture materials, yet certain guinea pigs in each series showed temporary febrile reactions or definite hemorrhagic lesions of the lungs indicative of a mild leptospira infection. Direct search for *Leptospira icteroides* in the blood of patients or in culture materials was not made because the dark-field microscope could not be used.

Subsequently, at Piura, the laboratory facilities were vastly improved, the use of the dark-field microscope was made possible by means of a storage battery, and a fresh stock of young healthy guinea pigs was received from New York, and fresh rabbit serum obtained in Piura. In the study of the materials obtained from five cases of yellow fever in Morropon all these added facilities were taken advantage of, with the result that the outcome was positive and convincing. Cultures from the five cases were examined after 11, 12, and 13 days, and in those from three cases living leptospiras were found.

By inoculation into suitable guinea pigs of culture material from these five cases, irrespective of whether or not leptospiras were detected under the dark-field microscope, a typical *Leptospira icteroides* infection was produced from four of the five cases. In one of these no leptospira had been detected in the culture tubes. Thus one case only yielded negative results, in that no leptospiras were found under the dark-field microscope and the animal inoculation was negative.

The leptospira was demonstrated in the blood or organ emulsions of the infected guinea pigs, and further transmission of each strain to other guinea pigs was obtained and pure cultures were secured.

A few points of practical significance appeared in the course of the present investigation. One is the importance of using fresh rabbit serum for culture media. Old rabbit serum, whether in pure form or incorporated with agar, etc., which had been kept for several months in a tropical climate, proved to be unsatisfactory for obtaining a growth of *Leptospira icteroides*. A second point of interest is the variation in susceptibility of guinea pigs to infection with *Leptospira icteroides*. In two of four series of positive animal inoculations with the Morropon culture materials only one-half of the guinea pigs inoculated with given materials developed typical symptoms. The other half either suffered from a transient mild infection, as evidenced by a few hemorrhagic foci in the lungs, or escaped infection altogether.

From these facts it is highly probable that the lung lesions and febrile reactions observed in certain guinea pigs inoculated with the Payta materials were due to a mild leptospira infection. In a comparative experiment the native guinea pigs procured in Payta were found to be more resistant to the leptospira infection than those recently brought from New York. In fact, only a small portion of the former succumbed to typical infection even when inoculated with a virulent strain of *Leptospira icteroides* obtained from the Morropon epidemic.

In conclusion it may be stated that of fourteen cases of yellow fever studied in Peru, a typical leptospira infection, together with the demonstration of the organism in experimentally infected guinea pigs, was obtained in four, while in the majority of instances indications of a mild, non-fatal leptospira infection were observed. In a few cases only were the results entirely negative.

The leptospira isolated from Morropon cases of yellow fever, which is morphologically and culturally identical with the Guayaquil and Merida strains of *Leptospira icteroides*, was also shown by immunity test to be indistinguishable from the Guayaquil organism.

IMMUNOLOGY OF THE PERUVIAN STRAINS OF LEPTOSPIRA ICTEROIDES.

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(Received for publication, August 7, 1920.)

In the course of a study of the etiology of yellow fever in Peru,[1] an effort was made to solve several related problems of the disease. The first point taken up was the behavior of the serums of Peruvian convalescents in Pfeiffer's reaction towards strains of *Leptospira icteroides* isolated elsewhere; the second concerned the action, *in vitro* and *in vivo*, on the Peruvian strains of the leptospira, of a polyvalent immune serum prepared in horses with several strains of *Leptospira icteroides* of Guayaquil origin; the third related to the difference in the natural resistance to the leptospira infection of the native guinea pigs as compared with guinea pigs recently brought from New York; and the fourth and last point bore on the availability of old rabbit serum for cultivating *Leptospira icteroides*. The last two questions had arisen as a result of the rather unfavorable outcome of the work carried out in Payta, and, as will appear later, proved to be important.

Pfeiffer Reaction with Serum from Convalescents.

The method was similar to that previously employed. 1 cc. of each serum was mixed with 0.2 cc. of a rich 6 week *icteroides* culture of Guayaquil Strain 1 and the Merida strain. The guinea pigs used were those recently brought from New York. Observations were made at the end of 1 hour.[2]

[1] Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 239.

[2] This work was done at the Belan Hospital, Piura, where we received cordial cooperation from Dr. M. Guzman R., Dr. A. Gonzalez, and Dr. Prieto, as well as the mother superior. We also wish to thank especially Dr. Marcos L. Vega, the government sanitary officer at Piura, for the arrangement to test the convalescents in Piura.

Nine convalescents were accessible for this test. Of these, four came from the Payta (1920), four from the Piura (1919), and one from the Morropon (1920) epidemic. The serums were obtained in the five 1920 cases within a period ranging from 7 to 36 days from the time of onset of the fever and in the four 1919 cases at the end of 10 to 11 months. The results obtained are shown in Table I. The interpretation of the findings is evident. All the Peruvian cases, except two instances to be discussed below, gave a positive Pfeiffer reaction to two different strains of *Leptospira icteroides*, irrespective of whether the case occurred in Payta, Piura, or Morropon.

TABLE I.

Pfeiffer Reaction with Serum from Convalescents.

Serum No.	Locality of epidemic.	Length of time between onset of yellow fever and withdrawal of blood.	Strain of *Leptospira icteroides*.	Results.
		days		
1	Payta.	7	Guayaquil No. 1	Positive.
2	"	7	" " 1	"
3	"	36	" " 1	"
4	"	17	" " 1	"
		mos.		
5	Piura.	11	Merida.	"
6	"	10	"	"
7	"	10	"	Doubtful.
8	"	10	"	Partial reaction.
		days		
9	Morropon.	21	"	Positive.
Control.			"	Negative.

The exceptions were the serums obtained from two persons who had had yellow fever 10 months previous to the time of testing, both giving a slight or partial reaction. It may be noted, however, that two other individuals who had had an attack 10 and 11 months previously reacted positively. The reason for a partial reaction in the first two cases probably relates to a gradual diminution of the antibodies responsible for the Pfeiffer reaction.

The foregoing observations are of value in establishing the identity of the organisms that were present in individuals suffering from yellow fever in Ecuador, Mexico, and Peru. The positive Pfeiffer

reaction with the serums of yellow fever convalescents in Payta also indicates that the guinea pigs of the Payta experiments which had shown suspicious febrile reactions and hemorrhagic lesions of the lungs and kidneys without developing a typical fatal infection, had actually suffered a mild infection with *Leptospira icteroides*. Finally, it may be added that the guinea pigs which were employed in the Pfeiffer tests and reacted positively showed no symptoms and survived, while the control animal (inoculated with the Merida strain) suffered from a typical but non-fatal infection.

Virulence.

The virulence of the Morropon strains was next tested on New York guinea pigs. The material used was an emulsion of kidney

TABLE II.

Determination of the Minimum Lethal Dose of Morropon Strains.

Amount of kidney emulsion in 1 cc.	Strain 1.		Strain 3.	
	Incubation period.	Result of inoculation.	Incubation period.	Result of inoculation.
cc.	*days*		*days*	
1	4	Died in 9 days.	No test made.	No test made.
0.1	4	" " 8 "	3	Died in 8 days.
0.01	3½	" " 7 "	4	" " 9 "
0.001	5	Recovered.	3	" " 7 "
0.0001	6½	Died in 11 days.	5	" " 8 "
0.00001		No infection.	5	" " 10 "

The animals used for this experiment served at the same time as controls for the therapeutic experiments reported in Table III.

made in saline solution in a ratio of approximately 1 gm. to 10 cc. After the coarse tissue fragments had settled, the turbid supernatant portion was employed for inoculation. Ascending tenfold dilutions were prepared with 0.9 per cent saline solution, and 1 cc. was injected intraperitoneally into guinea pigs, one animal being used for each dilution. Table II gives the minimum lethal dose as determined for Morropon Strains 1 and 3. The approximate minimum lethal dose for Strain 1 lies between 0.0001 and 0.00001 cc., and that of Strain 3 beyond 0.00001 cc. The grade of virulence of the strains is, therefore, about the same as that of strains previously studied.

Therapeutic Experiments with Anti-icteroides Serum.

The reaction of the Peruvian strains towards the anti-*icteroides* immune serum prepared with the Guayaquil strains confirms the other evidence of the identity of the organisms. It has been shown in our first paper[1] that the leptospira from Peru gave a positive Pfeiffer reaction with this immune serum. In the present series of experiments the object was to determine, in guinea pigs given multiple minimum lethal doses of Morropon Strains 1 and 3, the prophylactic and curative values of a specific polyvalent anti-*icteroides* immune horse serum. The immune serum was administered in varying quantities and at different periods after inoculation, even after the appearance of symptoms, up to the time when the animals were fast approaching the terminal stage of the disease induced. The guinea pig kidney emulsions used for infecting the animals were injected intraperitoneally in uniform amounts of 0.2 cc., this dose representing at least 2,000 minimum lethal doses of Strain 1 and 20,000 minimum lethal doses of Strain 3. The immune serum was given intraperitoneally also in doses of 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc., contained in saline solution to a total volume of 1 cc., and finally 1 cc. of undiluted serum. In order to obviate the factor introduced by an occasional unusually refractory animal, more than two of the recently brought guinea pigs were used in testing the effect of each dose of serum. Table III, which summarizes the results, brings out the following points. (*a*) The anti-*icteroides* serum is capable of checking the development of the infection, provided a sufficiently large quantity is given during the incubation period. The four deaths which took place among animals treated during this period occurred among those which did not receive serum until 72 hours after inoculation; *i.e.*, at the end of the incubation period. (*b*) A quantity of serum larger than 0.01 cc., given during the febrile stage (96 hours after inoculation) usually prevented the infection from reaching the icteric stage. This was true particularly with the animals infected with Strain 1, which had scarcely a tenth of the virulence of Strain 3. (*c*) The effect of the serum became uncertain when it was given during the icteric stage (5 to 6 days after inoculation). With 0.01 cc. no animal was saved, while 0.1 cc. saved three out of

four infected with Strain 3, but none with Strain 1. 1 cc. of undiluted serum saved one out of three with Strain 1 and three out of four with Strain 3. That the proportion of recoveries among the animals treated during the icteric stage does not correspond with the virulence of the strains can be explained only on the basis of the natural resistance of individual guinea pigs; at all events, death occurred in both groups, even when 1 cc. of serum was given.

TABLE III.

Protective and Curative Effect of Anti-icteroides Serum in Guinea Pigs Experimentally Infected with Leptospira icteroides.

Strain.	Amount of serum.	Animals treated before beginning of fever.			Animals treated during febrile stage.			Animals treated after jaundice appeared.		
		Total.	Remained well.	Died.	Total.	Recovered without jaundice.	Died.	Total.	Recovered.	Died.
	cc.									
0.2 cc. of Strain 1 culture, representing 2,000 minimum lethal doses.	0.0001	2	1	1						
	0.001	5	5	0	3	0	3			
	0.01	4	4	0	5	5	0	2	0	2
	0.1	4	4	0	3	3	0	2	0	2
	1				4	4	0	3	1	2
0.2 cc. of Strain 3 culture, representing 20,000 minimum lethal doses.	0.0001	2	2	0						
	0.001	6	5	1	2	0	2			
	0.01	6	4	2	5	3	2	2	0	2
	0.1	3	3	0	3	3	0	4	3	1
	1				2	2	0	4	3	1

The controls without serum treatment are represented by the animals of the experiment recorded in Table II.

Resistance of Native and Imported Guinea Pigs to Leptospira icteroides.

Certain tests made with guinea pigs obtained in Payta suggested that the native Peruvian guinea pigs are more refractory to the leptospira than guinea pigs brought from New York. To determine this point four groups of guinea pigs were inoculated, on May 16, 1920, with a uniform amount of kidney emulsion from a guinea pig which had shown a typical infection with Morropon Strain 4. The

animals inoculated comprised: (1) fifteen native guinea pigs which had once been inoculated with culture materials in Payta, without any definite infection having been induced; (2) six native guinea pigs which had not been used; (3) eight American guinea pigs which had formerly been inoculated with culture materials in Payta, without infection having been induced; and (4) four newly imported American guinea pigs. Table IV summarizes the result. It will be noted that the virulence and dose of the strain used were such that even the young American guinea pigs, used as a standard of susceptibility, did not all develop a fatal infection. This effect was rather an advantage, since, had multiple fatal doses been given, the difference in susceptibility might not have come out so clearly.

TABLE IV.

Relative Resistance of Native and American Guinea Pigs to Leptospira icteroides.

Guinea pigs.	Total No. injected.	Escaped infection (no jaundice).	Animals showing definite infection.*	
			Recovered.	Died.
Native (once used)	15	9	1	5
" (normal)	6	6		
American (old lot; once used)	8	3	3	2
" (new lot; normal)	4	0	2	2

* This includes guinea pigs which showed typical lesions when killed.

The striking feature of the foregoing experiment is the complete resistance of all six normal native guinea pigs in contrast to the four normal American animals, of which two, at least, died of a typical infection. Among the animals inoculated on a former occasion, both the native and the American groups contained susceptible as well as refractory ones, the difference being that a larger proportion of the native (60 per cent) than of the American (37.5 per cent) proved resistant.

The relatively large proportion of resistant animals among the native guinea pigs, as well as among the American guinea pigs which had withstood the unfavorable climate and other hardships, is a point to be taken into account in future work.

Deterioration of Rabbit Serum through Age.

That an alteration takes place in rabbit serum when it is subjected to long transportation in a hot climate is evident from the fact that no growth of *Leptospira icteroides* could be obtained with such serum. On the other hand, old rabbit serum which has been kept in the refrigerator up to 6 weeks has been found still suitable for making subcultures of *icteroides*. Whether the same would be true after 2 or 3 months standing at 4°C. has not, however, been determined.

The rabbit serum which was used in the cultivation experiments in Payta[1] was 3 months old and had been exposed to the ordinary tropical temperature of the region, which is sometimes 40°C. during the day, and it contained considerable precipitate. It was thought probable that the deterioration of the serum through age and climatic conditions might have been a factor in the unsuccessful outcome of the Payta experiments. Moreover, another lot of serum, which had been collected in New York in April and hermetically sealed, was brought down to Piura under ordinary temperature conditions. Some tubes contained precipitate, while others had remained clear. It was used when 50 days old for making subcultures of four Morropon strains of the leptospira, but when the culture tubes were examined 10 days later no growth could be found. Fearing a possible loss of all strains, we made two more sets of subcultures of the same strains, one set with the old and another with fresh rabbit serum. With the media containing the fresh rabbit serum a rich growth took place with three of the four strains, but there was no growth in the tubes prepared with the old serum.

Other factors, however, made it difficult to determine how important it is in general to employ fresh serum in culture work with *icteroides*. The Peruvian strains were unusual in that they early degenerated in culture. The initial cultures from human cases never became very rich, and they disappeared within about 3 weeks. The first generation cultures from the infected guinea pigs grew fairly well within a week, but at the end of 2 weeks examination showed only a few active organisms and many degenerating. Of the cultures of Strain 3, none was alive, and injection into guinea pigs failed to recover it. The remaining strains (Nos. 1, 2, and 4) were successfully

subcultured by using fresh rabbit serum, as noted above. It is certain that all the strains would have been lost if we had relied entirely on the old serum. The present study does not indicate whether the importance of using fresh serum applies only to the primary human and recently isolated generations or even to remote subcultures.

SUMMARY.

Serum from yellow fever convalescents from Payta, Piura, and Morropon gave a positive Pfeiffer reaction with the strains of *Leptospira icteroides* isolated in Guayaquil and Merida. The serum also protected the guinea pigs from these strains in the majority of instances. The Pfeiffer reaction was complete with all recent convalescents (7 to 36 days) but slight or partial in some instances with serum derived from individuals who had had the attack of yellow fever 10 months previously.

The virulence of the Morropon strains was found to be approximately the same as that of the Guayaquil or Merida strains. With one strain the minimum lethal dose for the guinea pig was less than 0.00001 cc. of a kidney emulsion from an infected guinea pig.

Suitable quantities of the anti-*icteroides* serum administered to guinea pigs inoculated with 2,000 to 20,000 minimum lethal doses of infective material prevented the development of the infection, or a fatal outcome, according as the serum was given during the incubation period or after fever had appeared. The earlier the administration of the serum the smaller was the quantity needed; during the incubation period 0.0001 to 0.001 cc. was sufficient, during the febrile period 0.01 to 0.1 cc. was required to check the progress of the disease, and even at the time when jaundice had already appeared, the injection of 0.1 to 1 cc. saved three out of four animals inoculated with Strain 3 and one out of three inoculated with Strain 1.

The native guinea pigs secured in Payta proved to be unusually refractory to infection with *Leptospira icteroides* as compared with normal guinea pigs recently imported from New York.

Fresh rabbit serum is recommended for culture work with *Leptospira icteroides*.

MIGRATION OF LYMPHOCYTES IN PLASMA CULTURES OF HUMAN LYMPH NODES.

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(*From the Department of Embryology of Carnegie Institution of Washington and the Department of Pathology, Johns Hopkins Medical School, Baltimore.*)

Plates 10 and 11.

(Received for publication, October 7, 1920.

In this study plasma cultures were made from normal and pathological lymph nodes obtained from operations at the Johns Hopkins Hospital. The method was as follows: Pieces were dropped into Locke-Lewis solution, and while in the solution were cut into small fragments 0.5 mm. or less in diameter. About 5 cc. of blood, withdrawn into a small paraffin-lined test-tube, were kept cool in a freezing mixture before centrifugalization. The centrifugalized blood was kept in the original tube surrounded by the freezing mixture and the plasma drawn off as the cultures were made. Cultures were made as rapidly as possible, in the usual manner, the number varying from 20 to 60 in the different series. Sometimes, however, 2 hours elapsed from the time the node was removed until the final cultures were made. The differences in time did not seem to affect the outgrowth from the explant. Both auto- and homoplasma were used, but no differences in the outgrowths were observed.

The lymphocytes were almost always the first cells to migrate out into the plasma clot. In autoplasma migration was observed within 1 to 2 hours, and in homoplasma $1\frac{1}{2}$ to $2\frac{1}{2}$ hours after the cultures were put in the warm box. The number of series of cultures was not sufficient to determine whether the initial latent period varies with the type of plasma and of tissue. The lymphocytes continue to migrate out into the clot for 2 or more days, or until practically all those which are living have left the explant. They migrate out into the plasma clot much more readily than onto the cover-slip over the liquefied areas. Liquefaction of the clot, as a rule, was delayed for

a day or two and varied greatly in extent. In some of the cultures however, the process began almost immediately after the cultures were made and in the course of an hour the explants were more or less surrounded by fluid. In these cultures lymphocytes were seen migrating out into the part of the clot which still remained attached to the explant. Comparatively few lymphocytes, on the other hand, migrated out onto the cover-slip over the liquid area. In some instances one portion of the clot was almost free from lymphocytes while another portion, attached to the explant, was full of them. Lymphocytes which came to the surface of the explant bordering the liquefied area fell to the bottom of this area, as many spherical lymphocytes were seen on the lower surface of the liquid.

The lymphocytes which appeared on the cover-slip over the liquefied area were generally associated with the large wandering cells (Figs. 6, 8, and 9) and with the giant cells. As the last two types of cells migrated out from the explant they occasionally carried with them one or more lymphocytes, the number sometimes being very large, which moved about on the under, free surface of the large cells and tended to remain there, rather than to wander off onto the cover-slip. Small lymphocytes could often be seen creeping around on a large giant cell, or moving back and forth in various directions, reaching the edge of the cell but rarely leaving it to proceed onto the cover-slip; they sometimes came to rest, assuming a rounded form, but still remained attached to the large cell. Apparently they adhered more readily to the flat wandering cells and giant cells than to the cover-slip. In fixed specimens the lymphocytes remained attached to these large cells (Figs. 8 and 9).

Lymphocytes sometimes tended to clump together in clusters of half a dozen or more. This clumping usually took place in the neighborhood of either small wandering cells or some foreign body. It resembled an agglutination without actual fusion of the cells. Only comparatively few of the lymphocytes tended to adhere together in this manner. The same phenomenon was observed in cultures (1) from normal nodes in both autoplasma and homoplasma, (2) from lymphadenitis specimens in autoplasma, (3) from tuberculous nodes in homoplasma, and (4) from metastatic sarcomatous nodes in homoplasma.

Paths of Migration.

In general, the lymphocytes migrated away from the explant and tended to spread out radially from it. When the route pursued by individual lymphocytes was observed in detail, however, it was found that they did not progress in direct radial lines away from the explant but moved in very irregular paths, as shown in Figs. 1 to 5. They sometimes made complete circles within a diameter of 0.033 mm. or less, or came almost to rest and turned and twisted about in a space of 0.006 mm. or less. They often migrated more or less rapidly for a few minutes, then came to rest, assumed a rounded form, and remained stationary for varying periods of time, from a few minutes to an hour or more. When the lymphocytes began to migrate again they did not always continue in the same direction. They crossed one another's paths without apparent deflection, or came into contact with one another without adhering. Contact with other cells or fibrous threads caused more or less deflection from the original course. It was not uncommon to see a lymphocyte come squarely against a strong fibrous thread with force enough to indent deeply the nucleus so as almost to divide it in two, remain there for a few seconds, and then slide off to one side or the other and continue its general course, the nucleus returning to its original form. The nuclei seemed semi-solid and very plastic, and continually changed shape as the cells met one obstruction after another; even when no obstruction was encountered, the nuclei underwent many changes in form as the cells twisted, turned, and elongated during migration. Sometimes the lymphocytes came up towards the cover, or penetrated into the depths of the clot, or migrated between the clot and the cover-slip; but as stated before, they generally migrated in the clot away from the explant. They did not appear to follow the fibrin threads but occupied the spaces between the latter. Migration from the explant continued until very few or only dead ones were left. There were always, of course, many more per cubic millimeter near the explant than towards the periphery of the clot, with occasional exceptions in old cultures.

TABLE I.

Series.	Distance traversed in successive min.	Rate of travel per min.		
		Maximum.	Minimum.	Average.
		mm.	*mm.*	*mm.*
(17) Carcinoma in homoplasma. 1½ hr. culture; 39°C. (Fig. 4).	(1) 8–4–13–9–8–2–5*	0.0173	0.0026	0.0093
(15) Normal node in homoplasma. 4 hr. culture; 38°C.	(2) 4–11–15–11	0.02	0.0053	0.0136
	(3) 11–13–10	0.0173	0.0133	0.0151
	(4) 9–15–13–4–7–9	0.02	0.0053	0.0113
(8) Metastatic sarcoma in homoplasma. 4 hr. culture.	(5) 18–12–12–18–12–17	0.0243	0.016	0.02
(12) Lymphadenitis specimen in homoplasma. 24 hr. culture; 38.5°C. (Fig. 3.)	(6) 4–15–6–6–7–12–9–8–9–12–17–12–8–9–5–14–21–13	0.028	0.0053	0.0133
	(7) 4–8–10–7–14–8–9–14–12–9–8	0.0186	0.0053	0.0126
	(8) 0–2–3–12–7–6–8–10–21–9	0.028	0.0026	0.0111
	(9) 8–12–8–5–14–9–6–10–13–9–9–8	0.0186	0.0066	0.0125
	(10) 5–7–6–10–5–6–2–9–9–7–11	0 0146	0.0026	0.0093
	(11) 18–15–12–?–?–?–11–15–12–22–7–8	0.0293	0.0093	0.0178
	(12) 8–5–17–5	0.0227	0.0066	0.0111
(16) Normal node in homoplasma. 24 hr. culture; 39–40°C.	(13) 8–7–10–9–8–8–8	0.0133	0.0093	0.0109
	(14) 8–5–9–8–7–6–8–7	0.0120	0.0066	0.0152
	(15) 4–10–5–8–10–13–6–5–8–11	0.0173	0.0053	0.0106
	(16) 17–11–12–6–15–18	0.024	0.008	0.0173
	(17) 7–12–3–8–4–10–3–5–11–8–5–6–15–12–11–8–5–9	0.02	0.004	0.0106
(13) Normal node in homoplasma. 20 hr. culture; 37.5°C. (Fig. 2).	(18) 6–9–6–5–4–7–5–0–0–0–8–6–3–6–7–7–10–3–6–6–2–7–2–8–5–0–0–0–0–2–2–2–1–8–6–7–0–0–0–0–5–5–0–0–0–0–0–0–2–4–4–5–6–0–0–0–0–0–0–0–	0.0133	0.0013	0.0069
(18) Tuberculous node in homoplasma. 48 hr. culture.	(19) 8–21–16–10–15–14–8–12	0.028	0.0106	0.0173

* The figures for the distance traversed are given in millimeters multiplied by 750.

TABLE I—*Concluded.*

Series.	Distance traversed in successive min.	Rate of travel per min.		
		Maximum.	Minimum.	Average.
		mm.	*mm.*	*mm.*
(8) Metastatic sarcoma in homoplasma. 48 hr. culture; 38.5–39.5°C.	(20) 8–14–12–12–?–?–0–0–0–0 for 30 min.	0.0186	0.0106	0.0152
	(21) 10–12	0.016	0.0133	0.0146
	(22) 9–8–8–7–10–7	0.0133	0.0093	0.0113
	(23) 18 (Fig. 1)	0.025		
	(24) 12–10–10–6	0.016	0.008	0.0126
	(25) 10–9–6–3	0.0133	0.004	0.009
(12) Lymphadenitis specimen in homoplasma. 44 hr. culture.	(26) 7–15–15–14–11	0.02	0.0093	0.0165
	(27) 13–1–5–11	0.0173	0.0013	0.01
(9) Tuberculous node in autoplasma. 4 day culture (Fig. 5).	(28) 6–6–5–5	0.008	0.0066	0.007
	(29) 5–5–5–4–2–3–5–7	0.0093	0.0026	0.006
	(30) 5–8–2–9–8–11–6	0.0146	0.0026	0.0093
	(31) 5–5–2–6–6–10	0.0133	0.0026	0.0096
(18) Tuberculous node in homoplasma. 7 day culture; 37.5°C.	(32) 10–12–9–6–5–8–3–4–3–8	0.016	0.004	0.0077
	(33) 7–5–6–8–11–11–10–12	0.016	0.0066	0.0116
	(34) 10–13–12	0.0173	0.0133	0.0154
	(35) 5–4	0.0066	0.0053	0.006

Rate of Migration.

The rate of migration was studied by camera lucida drawings made at minute intervals with a magnification of 750 or 1,250 diameters. Most of the lymphocytes migrated out of the field of the camera in a few minutes, so that the period of observation for any one lymphocyte was usually rather short. There was great irregularity in the distance covered during successive intervals. The greatest distance traversed during 1 minute was 0.03 mm., the maximum distances for the different lymphocytes varying from, 0.03 to 0.006 mm. The minimum varied much more; namely, from 0.013 to 0.0013 mm. per minute. This was to be expected, since lymphocytes often slowed down before they came to rest. The average rate varied from 0.02 to 0.006 mm. per minute. Determinations were made in cul-

tures from $1\frac{1}{2}$ hours to 7 days old, but no especial differences were noted in the rate of migration. Measurements were also made of lymphocytes (1) from normal nodes in homoplasma, (2) from lymphadenitis specimens in homoplasma, (3) from tuberculous nodes in auto- and homoplasma, (4) from metastatic sarcomatous nodes in homoplasma, and (5) from a metastatic carcinomatous node in homoplasma, but the rate of migration was essentially the same in all, as shown in Table I. The observations were not extensive enough to draw conclusions as to the meaning of the variations in rate in cultures of different ages or in the different combinations of plasma and explant. We have not been able to discover any differences in lymphocytes from normal and pathological nodes.

Hirschfeld[1] in 1901 observed the ameboid activity of lymphocytes from a case of lymphatic leucemia, using Deetjen's method by placing a drop of leucemic blood on a film of 1 per cent agar which contained 0.6 gm. of sodium chloride, 6 to 8 cc. of a 10 per cent solution of sodium metaphosphate, and 5 cc. of a 10 per cent solution of dipotassium phosphate, per 100 cc.

Structure.

The appearance of the cytoplasm of the living lymphocyte differed from that of the other cells. This was more noticeable in the elongated moving lymphocyte. It is difficult to describe other than that it was darker and less transparent. The cytoplasm seemed to be rather firm in consistency, since it rarely spread out into a very thin layer like that of the wandering cells. The lymphocytes in fresh cultures contained very few granules, and with neutral red only two or three granules at most became red. In older cultures there was a gradual increase in the number of granules which took up the neutral red. They were always small and did not seem to have vacuoles about them. The mitochondria were difficult to see unless colored with Janus green or Janus black No. 2. They were few in number, granular in form, and small. Lymphocytes often contained one or more fat globules (Fig. 1). In the migrating lymphocytes one could usually distinguish a homogeneous ectoplasm and a darker endoplasm which contained the granules and mitochondria.

[1] Hirschfeld, H., *Berl. klin. Woch.*, 1901, xxxviii, 1019.

The nucleus had a peculiar waxy appearance; it appeared to be homogeneous or structureless, and the nucleolus was very difficult to discern.

Shape.

The resting lymphocytes (Figs. 1, 6, and 7), and those that had fallen to the bottom of the liquefied area, were usually more or less spherical and presented the characteristic picture of a thin shell of cytoplasm enclosing a comparatively large nucleus eccentrically placed. The migrating or moving lymphocytes, on the other hand, had a peculiar, elongated shape, with the nucleus always at or very near the anterior or forward-moving end (Figs. 1 to 5 and 8 to 10). The cells seemed to possess a distinct polarity. The degree of elongation varied during successive minutes, but the nucleus was always near the anterior end with a small amount of ameboid cytoplasm in front which was continually changing shape and was sometimes so small in extent as to be scarcely visible. This anterior cytoplasm was homogeneous and free from granules. There was usually very little cytoplasm at the side of the nucleus; the bulk of it, in these migrating cells, lay behind the nucleus and was more or less finger-shaped, consisting of a thin ectoplasm enclosing an endoplasm which contained mitrochondria and granules. The nuclear region was often the broadest part of the cell, but the nucleus itself was very plastic and was continually changing shape, although it seemed to be more solid than the cytoplasm.

When the lymphocytes came to rest and assumed a rounded form, the large cytoplasmic tail formed the thicker part of the cytoplasmic rim and forced the nucleus into a slightly eccentric position; when the lymphocytes began to move and stretched out, this thickened part of the cytoplasmic rim formed the elongated tail. The resting as well as the migrating cells possessed a distinct polarity.

We were not able to distinguish a centriole or a central area as in the mesenchyme cells of chick embryos, so that it was impossible to determine whether polarity had any relation to a centriole. The living lymphocytes in the plasma clot were remarkably constant in size, shape, and structure, and showed no tendency toward transition to other types of cells. They were easily recognized, either at rest

or during migration, and their general appearance was so distinctive that they were never confused with the other types. Occasionally a large mononuclear cell was seen which resembled a lymphocyte in every particular except size. The general appearance of the cytoplasm and nucleus was similar. They migrated with the nuclear end first and were then elongated, but assumed a more or less rounded form when at rest (Fig. 4).

In fixed and stained preparations the lymphocytes found on the surface of the large wandering cells and giant cells, and more rarely on the cover-slip, showed a greater diversity of form than the living ones in the plasma clot (Figs. 8 to 10). This was probably due to the peculiarities of the environment during life, where they had a more or less flat surface to which they could adhere and upon which they could spread out.

Neither mitosis nor amitosis was observed in lymphocytes. All the lymphocytes studied were mononuclear.

SUMMARY.

1. Lymphocytes were usually the first cells to migrate out into the plasmic clot from explanted pieces of lymph nodes.

2. Their paths of migration were irregular but in general they proceeded away from the explant.

3. The lymphocytes migrated at rates varying from 0.03 to 0.0013 mm. per minute. The rate of any one varied from minute to minute, and they often came to rest for varying lengths of time.

4. The migrating lymphocytes were very much elongated, with the nucleus always near the anterior end. The elongated tail contained the endoplasm with a few granular mitochondria and usually a few granules which took up neutral red.

5. The lymphocytes in cultures made from normal and pathological lymph nodes in auto- and homoplasma showed no differences.

EXPLANATION OF PLATES.

PLATE 10.

FIG. 1. 48 hour culture from a metastatic sarcomatous node in homoplasma. A migrating lymphocyte (No. 23, Table I) at ½ minute intervals. The change from the migrating form to the resting form and back to the migrating is shown. Distance traversed during the minute 0.025 mm. × 1,250.

FIG. 2. 20 hour culture from a normal node in homoplasma. A migrating lymphocyte (No. 18, Table I) drawn at 1 minute intervals for 1 hour. The broken arrow points to the explant. × 750.

FIG. 3. 24 hour culture from a lymphadenitis specimen in homoplasma. Migrating lymphocytes in the same field drawn at 1 minute intervals. × 750.

FIG. 4. 1½ hour culture from a metastatic carcinomatous node in homoplasma. A migrating lymphocyte (No. 1, Table I) and a large lymphocyte drawn at 1 minute intervals. × 750.

FIG. 5. 4 day culture from tuberculous node in autoplasma. Four migrating lymphocytes in the same field (Nos. 28, 29, 30, and 31, Table I) drawn at 1 minute intervals. × 750.

PLATE 11.

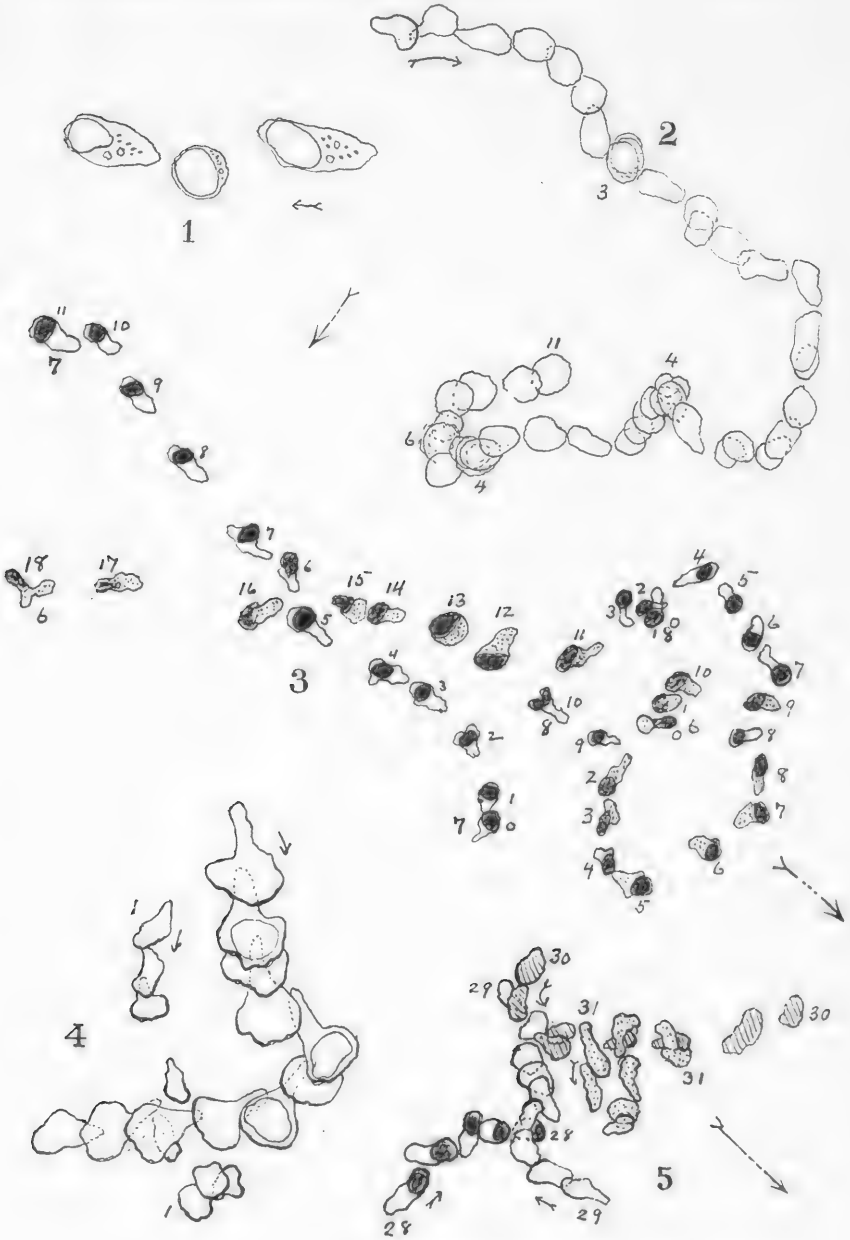
FIG. 6. 7 day culture from a normal node in autoplasma. Four resting lymphocytes and two large wandering cells on the cover-slip. Neutral red and Janus green were added at the time of observation. The lymphocytes showed neutral red granules and green mitochondrial granules. The large wandering cells had a deep red center (dark granular in the figure) near the nucleus, surrounded by an extensive area of fat globules. Mitochondria were abundant about the fat area and extended into the clear ectoplasm. × 750.

FIG. 7. 96 hour culture from a metastatic sarcomatous node in homoplasma. Two resting lymphocytes showing neutral red granules. Neutral red was added at the time of observation. × 1,250.

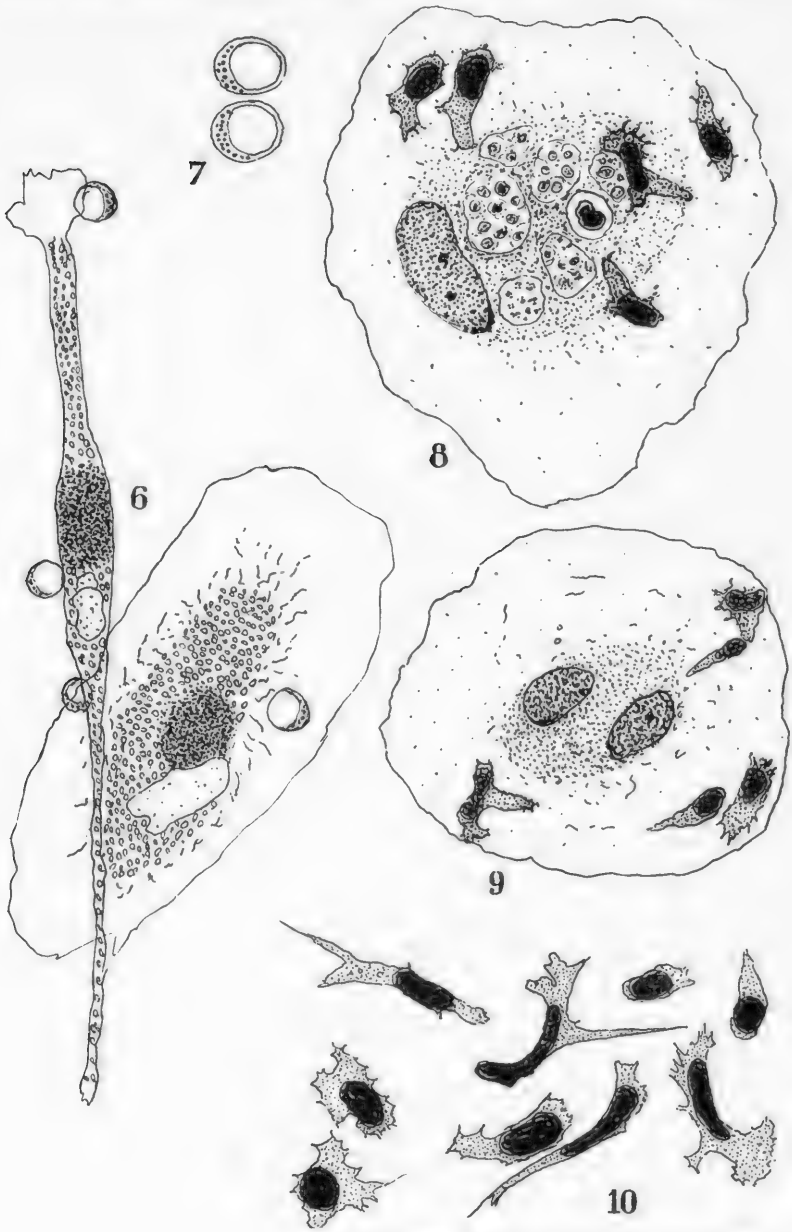
FIG. 8. Fixed and stained specimen from a 48 hour culture of a tuberculous node in homoplasma. Five migrating lymphocytes are shown on the surface of a large mononuclear wandering cell which is spread out on the surface of the cover-slip. The wandering cell contains dead lymphocytes in various stages of digestion. × 1,250.

FIG. 9. Five migrating lymphocytes on the surface of another large wandering cell from the same culture as Fig. 8. × 1,100.

FIG. 10. Nine migrating lymphocytes from the surface of other wandering cells from the same culture as Fig. 8. × 1,250.



(Lewis and Webster: Plasma cultures of human lymph nodes.)



(Lewis and Webster: Plasma cultures of human lymph nodes.)

STUDIES ON ENDOTHELIAL REACTIONS.

IV. The Endothelium in Experimental General Miliary Tuberculosis in Rabbits.

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Plate 12.

(Received for publication, September 21, 1920.)

It is the purpose of this paper and the others of the series (Foot, 1919, 1920, *a*, *b*) to draw attention to the important part taken by cells, originating in capillary endothelium, in various types of inflammation. In the first paper (Foot, 1919) their reaction to the presence of a foreign body (agar-agar) was discussed; in the two succeeding their behavior in experimental subcutaneous and pulmonary tuberculosis in the rabbit was described. By means of intravenous injections of a colloidal suspension of carbon it was shown that the cells forming the foreign body tubercles, as well as those found in lesions of experimental tuberculosis, were traceable to the endothelium of the neighboring capillaries. The present paper is a report of the histogenesis of the cells forming the tubercles produced in rabbits by the intravenous injection of tubercle bacilli. Such a general infection as this simultaneously produces lesions in various organs, and the question arises whether the cells found in all these are traceable to the capillary endothelium.

The literature on experimental tuberculosis has already been discussed at length in the earlier articles of the series (Foot, 1920, *a*, *b*), but it may be well to review it briefly. Sewell, Watanabe, and Wechsberg have experimented upon the lung, studying the lesions produced by the insufflation of tubercle bacilli through the trachea, with or without the use of a vital staining method.[1] The

[1] A recent article by Permar (Permar, H. H., *J. Med. Research*, 1920, xlii, 9) has appeared since the present paper was submitted for publication. It is interesting to compare his findings in experiments on intratracheal injections of carmine in guinea pigs and rabbits, coincident with intravenous administration of isamine blue, with those of this paper and of Paper III (Foot, 1920, *b*). His conclusions as to the endothelial origin of the phagocytic cells of the lung tissue coincide in every way with mine and his emphasis of the non-participation of the epithelium in these phenomena of phagocytosis seems well supported by facts.

liver has been extensively studied because of the readiness with which exact amounts of bacilli can be introduced into the intramesenteric circulation and thence into the portal vein and its radicles in the liver. Among others Kockel, Miller (1902), Oppenheimer, Goldmann, Evans, Bowman, and Winternitz, Kiyono, and more recently Soper have investigated the histogenesis of tubercles produced by the intraportal injection of tubercle bacilli. Kostenitsch and Wolkow in 1892 studied tuberculous lesions produced in the cornea and kidney of the rabbit by means of local injections. So far as can be ascertained there are no reports on experimental work on miliary tuberculosis, in which methods of vital staining have been brought to bear.

Since the consensus of opinion has not been in favor of the endothelial origin of the so called epithelioid cell, particularly in the case of the lung and kidney, the following experiments were performed which confirm the theory that the tubercle in the lung and kidney is not of epithelial origin.

Two vital stains were employed, one a benzidine dye—Niagara blue 3 B[2]—the other a colloidal suspension of carbon in the form of Higgins' waterproof India ink, as in the preceding experiments. This is similar to the suspension devised by McJunkin and more easily prepared. The former gives a granular vital staining reaction, quite similar to that obtained with trypan blue, in the macrophages of Metschnikoff (a class of phagocytes designated by a large variety of names, but undoubtedly of endothelial origin), the lymphoid reticulum, the endothelium of the liver sinusoids, which is known as Kupffer cells, the fibroblasts, and the epithelium of the convoluted tubules of the kidney and, to a slight degree, that of the liver. It also appears in the polymorphonuclear neutrophils under conditions of extravascular existence or circulatory stagnation. Necrotic tissue and occasionally elastic fibers take on a diffuse blue color in its presence. Higgins' ink is chiefly taken up *in situ* by the endothelium of the smaller capillaries in certain organs, but it is also phagocytosed by the endothelium anywhere in the presence of inflammation. The free endothelial phagocytes likewise take it up in large quantities; if a benzidine dye is also present granules of carbon and this dye are taken up by these cells at the same time.

[2] The Niagara blue 3 B was kindly furnished by Dr. George B. Wislocki, of the Department of Surgery of Harvard Medical School.

Therefore in an experimentally produced miliary tuberculosis in which both of these dyes are present, the epithelioid cells will contain ink only if they are the product of the only tissue that takes it up; that is, the endothelium. It was of particular interest to determine whether this would be true in the lesions produced in the spleen, lymph nodes, and omentum, where many phagocytes are present and where the reticulum, on the one hand, and the mesothelium, on the other, have been considered the parent tissues of the epithelioid cells. It would be well to know also whether or not the renal epithelium contributes to the tubercles in the kidney, as claimed by Kosteritsch and Wolkow.

By using the two dyes together it is possible to obtain a relatively exact differential staining reaction; the macrophages, lymphoid reticulo-endothelial cells, and the mesothelium, and the convoluted tubules of the kidney show a marked affinity for Niagara blue. Although many of these cells take up ink the renal tubular epithelium never does so, and the others always show more blue than black granules in the presence of both dyes.

Soper has quoted the experience of other observers, as well as himself, to show that the epithelioid cell loses its affinity for benzidine dyes once it has taken up tubercle bacilli, or has come under their influence. In studying the formation of subcutaneous tubercles I (1920, *a*) have found this to be in a measure true, as the cells seem to become vacuolated and the dye granules dispersed and lost after tubercle bacilli are phagocytosed. It does not seem that cells loaded with these dyes, for example lymphoid reticular cells or omental macrophages, would immediately lose all the dye in the presence of tubercle bacilli, nor do the findings of this experiment indicate that such is the case.

Rabbits have, therefore, been injected intravenously with bovine tubercle bacilli and at the same time been vitally stained, the resulting lesions being studied to determine the origin not only of their epithelioid cells, but also of the so called tubercle reticulum. By combining the vital and the usual connective tissue stains it is hoped that new light may be shed on the origin of this fibrillar structure, particularly by observing its formation in organs normally poor in collagen fibrils. Kon, and Yoshida, working with Rössle, and more recently Warren have demonstrated the possibility of its being produced by the endothelium. Downey has discussed the literature on this

subject in connection with the formation of the fibrils of the lymphoid reticulum. Hueck's recent communication further strengthens this view which is discussed more fully below.

Technique.

Four rabbits were given intraperitoneally 10 cc. of a 1 per cent solution of Niagara blue 3 B (twice purified) in distilled water. The next day this injection was repeated, with a 1 per cent solution of the commercially pure dye, which is less concentrated. The 3rd day the injection with the weaker dye was repeated and 5 cc. of Higgins' waterproof ink and distilled water, in equal parts, were introduced into an ear vein. The animals were now, by this means, vitally stained; their skin and mucosa were a deep ultramarine. On the 4th day each was given 1 cc. of a suspension of bovine tubercle bacilli intravenously, the strain employed being one of those used and tested out in previous experiments (Bov. XIV), and the dilution such that 1 cc. equaled 1 mg. of the bacilli. The suspension was made by rubbing up a weighed amount of culture from glycerol agar slants in normal salt solution, by means of a glass bulb pestle, shaking in a machine, and diluting and rediluting with normal saline solution until the desired concentration was reached. Intraperitoneal injections of 10 cc. of 1 per cent twice purified Niagara blue and intravenous injections of 5 cc. of 50 per cent Higgins' ink were instituted 3 days later and administered twice a week until the animals died or were killed. This kept all the rabbits vitally stained, with one exception. Rabbit 1 was killed 7 weeks after the experiment was begun, but the injections were only continued for 4 weeks, in this instance, in order to see what would become of the dyes accumulated in the tissues during that time. Later two other rabbits were given one intravenous injection of 1 mg. of the same strain of tubercle bacilli and the dyes were withheld until the 3rd week, when they were administered in exactly the same way as in the first set and continued until the animals were killed. The object was to begin supplying the dyes to the tissues at the approximate onset of the disease, instead of simultaneously with the inoculation.

This lot of six rabbits gives a series of potential cases of miliary tuberculosis representing stages of 1, 2, 2½, 4, 5, and 7 weeks development. All except one were killed by injecting 4 per cent neutral formaldehyde into the beating heart under anesthesia. Rabbit 2 died of an intercurrent coccal infection after 2 weeks, but showed a few pulmonary tubercles. The tissues were fixed both in neutral 4 per cent formaldehyde and in Helly's fluid (potassium bichromate 2.5 gm., corrosive sublimate 7 gm., water 100 cc., and neutral 40 per cent formaldehyde 10 cc.) in order to compare the action of these fixatives on the intracellular granules of Niagara blue. Helly's fluid is found to be in every way superior to neutral formaldehyde alone; the granules are somewhat paler than when only formaldehyde is used, but as they are apt to be very dark in the latter case, this is a distinct advantage as it enhances the blue color and prevents confusion between

this dye and the ink. Sodium hyposulfite should not be used to remove the iodine, before the final staining, as it tends to bleach the dye completely; 95 per cent alcohol, although slower, is preferable, as it does not affect the vital stain in any way.

Sections of 5 microns were cut in paraffin and stained with Mayer's aqueous carmalum (ammonia alum 20 gm., carminic acid 2 gm., and distilled water 400 cc.), with Mallory's phosphotungstic acid-hematoxylin and aniline blue connective tissue stain, and with Delafield's hematoxylin in conjunction with the Ziehl-Neelsen carbolfuchsin stain. It is, of course, necessary to rely upon a red nuclear stain for the routine procedure, in order to get the best contrasts with the blue vital dye; there is too little difference in color between this and hematoxylin or methylene blue to render them available except for topography or for photographic purposes. Sections were also impregnated by the Bielschowsky-Maresch method. Six organs were chosen for detailed study as described below.

Pulmonary Lesions.

Since bacilli, after being introduced into the circulation, are first swept into the pulmonary capillaries and, as Miller (1919–20) has shown, into the intrapulmonary lymph nodules, lesions are found developing in this organ before they can be found elsewhere. After 1 week two types of tubercle are distinguishable. The first consists of small aggregations of deeply ink-stained cells, 70 microns in diameter, which in the light of their further development, seem to be merely an agglomeration of phagocytic cells in response to the presence of the ink. The second type is composed of true, specific tubercles, few in number and only found after some searching. They show no Niagara blue, but their epithelioid cells contain ink globules. After 17 days development larger tubercles, about 375 microns in diameter, can be found. They show a few syncytia, or giant cells, and contain a uniform amount of ink in the cells composing them. Polymorphonuclear leucocytes now appear at the periphery of the tubercles. The latter are obviously interstitial in origin and are situated near the blood vessels of larger caliber; one occasionally finds crescentic thickenings of the endothelium of these vessels, the crescents being deeply impregnated with ink globules. Similar lesions, which are not crescentic, are found in the intrapulmonary lymph nodules, near bronchioles. The alveolar spaces are not involved until surrounded by the tuberculous infiltrate, when they begin to fill up with the large, emigrated endothelial cells described in the preceding paper (Foot,

1920, *b*). These cells, like those of the tubercles, contain fine granules of carbon.

After 4 weeks development the tubercles are visible to the naked eye, being 0.3 to 0.7 mm. in diameter and occupying the walls and air sacs of the lobules affected. The tubercles often include larger vessels and cause their obliteration by overgrowth of the lining endothelium, which proliferates not only into the crescentic masses just described, but becomes a plug of anastomosing, rather stellate cells, not unlike the embryonal mesenchyma in appearance. Whether these lesions start within the vessels and work outward, or whether they surround and involve the vessels from without, is sometimes difficult to determine. As the infection is presumably intravascular, in this experiment, the first hypothesis is the more plausible. All the cells composing the tubercles are dotted with ink globules and a few now show granules of Niagara blue. Caseation begins at the center of the larger lesions, the caseous mass taking on a diffuse bluish stain. Polymorphonuclear leucocytes penetrating the tubercles also show a granular blue vital stain, but no ink. This indicates that the blue dye is available here, as elsewhere in the body and that it has no particular affinity for the epithelioid cells, whereas the ink has. As in the experiments on intratracheal infection no activity is seen among the epithelial cells, other than regenerative phenomena. A light vermilion blush now suffuses the tubercles in the Van Gieson preparations, while a similarly faint bluish tinge appears in those stained with Mallory's connective tissue method. This can be seen to be due to the appearance of delicate fibrils in the lesions, not only among fibroblasts but also in and between the ink-containing epithelioid cells. These fibrillæ are also demonstrable as pinkish threads with the phosphotungstic acid-hematoxylin stain. While they are always red with the Van Gieson process, they may be red or reddish with Mallory's connective tissue stain, or blue or purplish with the phosphotungstic acid-hematoxylin. This would, presumably, indicate that they were gradually becoming impregnated with collagenous material.

After 5 weeks all these processes are more striking. The fibrous reticulum is now well formed and easily seen, the caseation more advanced and a deeper blue. After 7 weeks the lung is so widely

involved as to leave little normal tissue. In this case, as noted under Technique, the use of the dyes was discontinued in the 4th week; consequently the newer tubercles are almost devoid of ink and Niagara blue, while the older ones show plenty of both, the latter in diffuse form in the caseous areas. Older tubercles are sometimes centrally stained and peripherally free from the vital dye, the younger portions of the lesion having apparently formed after the supply of available dye was exhausted. There are a great many intravascular, crescentic, and deeply ink-stained tubercles in the sections from this rabbit, as well as a few of the obliterating, retiform type.

Thus it is found in the lung that the tubercles develop in and near larger vessels, that they contain much ink, but little granular Niagara blue, and that the reticulum is formed chiefly in the epithelioid cells. Groups of migrating endothelial cells lying free in the alveoli can be seen in the 5 and 7 week preparations, in which delicate fibrils are demonstrable. There is no question of any relation between these cells and the alveolar walls, as their origin has been worked out and reported in the preceding paper (Foot, 1920, *b*). The fibrils first form about the periphery of the cells by consolidation of their cytoplasm and cell processes (ectoplasm); next there is vacuolation of the endoplasm and a consolidation of the walls of the vacuoles, with the production of fibrillar circles and lines, which gradually seem to twist and break into fibrillæ. The process is similar to that described by Hueck in his article on the mesenchyma. This is not strange in view of the embryonal origin of these cells.

Sections from the lung after 5 weeks were impregnated with silver by the Bielschowsky-Maresch method with the view of obtaining an idea of the distribution of the reticulum fibers among the epithelioid cells. Fig. 1 shows that they are readily demonstrable in cells that contain carbon particles. The majority of the epithelioid cells were found to be free from reticulum fibers, but there are many places in the sections where the fibers can be found running in and among these cells and forming a fairly close network about them. Of course, many fibers run out from the alveolar walls into the exudate, but there are instances in which no connection between these and the smaller fibrils in the epithelioid cells is demonstrable. This method of impregnation, then, bears out what has already been noted with the connective tissue stains.

Hepatic Lesions.

In the hepatic lesions also, distinction should be made between small pseudotubercles, apparently due to the presence of ink, and the true tubercles resulting from the infection. During the first 3 weeks there are slight changes in the sinusoidal endothelium, in part probably due to the ink, in part possibly the forerunner of true tubercles; but it is not until the 4th week that fully developed, specific lesions appear. They seem to begin as syncytia and to be situated in sinusoids lying in the mid-zone, between the hepatic and portal systems, usually nearer the former. The cells forming them are so heavily laden with ink globules that it is difficult to make out their histology (Fig. 2). The ink is usually arranged peripherally in the syncytia and single cells, forming a dense black rim that occupies much the same portion of the cytoplasm in the former, as that in which the nuclei are generally grouped, while in the latter it forms a ring about the single nucleus. In no organ is it so plentiful in the cells as in the liver. There is no indication that the syncytia are formed by mitotic nuclear division, without cytoplasmic division, as claimed by Soper; it seems more likely that they are the result of a fusion of several single cells. This is the rule in the subcutaneous lesions, and there is no apparent reason why there should be an exception in the liver endothelium. After 5 weeks the tubercle reticulum is demonstrable in the sections stained by appropriate methods. The fibers are best seen if green or greenish brown filters are interposed between the condenser and slide, when they stand out sharply. Here, as in the lung, they can be definitely demonstrated in the epithelioid cells, as well as in the few connective tissue cells present.

Splenic Lesions.

In the preparations representing the first few weeks development nothing definite can be distinguished. The pulp and its phagocytes are intensely blackened with ink, and the splenic reticular cells are deeply stained with the blue dye, taking up the ink to a much less degree. In the 4 week preparations the tubercles are mature and situated at the periphery or near the center of the Malpighian corpuscles. Their epithelioid cells contain a varying amount of ink

but far less than heretofore encountered in experimental tubercles of this type. Only a few of them contain Niagara blue granules. Small tubercles forming in the secondary nodules of Flemming show much ink in their cells. The tubercle reticulum is beginning to form.

After 5 weeks, caseation is found and the caseous material is stained a diffuse blue. There is the same scanty deposit of ink in the tubercles (Fig. 3) of the Malpighian corpuscles but a moderate amount in the secondary nodules. Although the splenic reticular cells are deep blue, only a few of the epithelioid cells show this stain. After 7 weeks, however, the tubercles are deeply stained with ink. Besides the tubercles, one finds groups of ink-bearing cells in the lymphoid nodules; they show no tubercle reticulum, while the true tubercles exhibit a fully formed network of fibrils. In this way the two are readily distinguishable. The splenic sinuses are widely dilated and almost free from ink-bearing cells; it seems as if these had migrated to the lymphoid tissue and, possibly, into the tubercles as well. The reticular cells of the splenic tissue are still deeply stained with Niagara blue.

Thus, although the splenic sinuses and their free cells are so heavily laden with ink whenever it is administered intravenously, relatively little of the ink seems to be found in the cells forming the tubercles located in the lymphoid tissue of the Malpighian corpuscles, while a moderate amount is present in the smaller, or secondary nodules. This is not readily explained; the subject will be discussed further in the consideration of the lesions in the lymph nodes, where a somewhat similar condition exists.

Renal Lesions.

Definite tubercles are first found in the sections from the 4 week rabbit although there are indefinite changes in the glomerular tufts and the walls of the vasa recta before this time. The tubercles vary from 75 to 750 microns in size and appear to be of two types, a moderately diffuse infiltration of the interstitial tissue between the cortical tubules, and more localized collections of epithelioid cells, with an underlying glomerular topography—one can make out the remains of Bowman's capsule with swollen cells surrounded by the tuberculous lesion. In the first type, there are many small endothelial

leucocytes, similar to those seen in the subcutaneous lesions described in the first and second papers of this series (Foot, 1919, 1920, *a*). These are compact cells, with a bean-shaped nucleus and rather dense cytoplasm, about half the size of fully developed epithelioid cells, many of which are found in these tubercles. These cells often contain ink globules, as do their syncytial derivatives. The tubular epithelium, on the other hand, takes up no ink, although that of the proximal convoluted tubules is vitally stained with Niagara blue. It is significant that the tubercles show ink and that their cells take on no blue stain, except in scattered places where degeneration is obvious. One can find mitotic figures in the epithelium of the surrounded tubules, but this is probably an attempt at regeneration, as they are found only in cells that are still *in situ* in the tubules. In some of these diffuse tubercles it is possible to see the tubular epithelium degenerating and disintegrating, with endothelial cells, containing ink, pressing in upon its thinned out portions and apparently producing pressure atrophy.

After 5 weeks the tubercles are more numerous, larger, and show beginning central necrosis, with the usual diffuse blue stain in the caseous areas. The two types of tubercles, glomerular and interstitial, can still be distinguished. Whereas the tubercles were formerly all cortical, there are now a few of the interstitial type among the collecting tubules and Henle's loops in the medulla. They do not differ from the cortical interstitial type and their cells show ink (Fig. 4), but no Niagara blue. After 7 weeks the picture is not noticeably altered, but is merely more pronounced. The tubercle reticulum is demonstrable after the 4th week and is best seen in the 7 week lesions, differing in no way from that of the lesions in other organs.

That many of the tubercles originate in the glomeruli is indicated by the following facts. (1) Remains of glomerular structure are present in some of them. (2) The early tubercles are often rounded and of the same diameter as the average glomerulus. (3) The tubercles occur chiefly in the cortex. (4) The glomerulus is the chief renal structure showing ink in its cells; moreover, there is none in the tubules. Nevertheless, there are other lesions showing no evidence of glomerular architecture which occur near the vasa recta and may be found well down in the pyramids where no glomeruli are located. Ink is found in the endothelium of the vasa recta in controls. It

seems probable, then, that the tubercles originate from two sources, the endothelium of the glomerular capillaries and that of the capillary branches of the vasa recta, the former remaining near their site of origin, the latter migrating into the interstitial connective tissue between the tubules. There is no evidence that the epithelium takes an active, formative part in the process, although a few epithelial cells may be included in the tubercles and survive there for a time. Such cells, which have their origin in the vitally stained convoluted tubules, may contain granules of Niagara blue, and their nuclei often stain a bright blue, which is pathognomonic of degeneration and indicates early karyolysis. The ink globules prove to be an invaluable indicator of the origin of the component cells of the tubercles in the kidney as elsewhere.

Omental Lesions.

The omentum, bathed as it is in a copious supply of Niagara blue from the intraperitoneal injections, shows deeply stained mesothelial cells on the surface, some staining of the reticulum of the taches laiteuses, and a double staining of the wandering macrophages in its meshes. These macrophages contain some ink as well as the blue granules of the benzidine dye. The vascular endothelium of the complex capillary network, however, is dotted with black spots alone. The tubercles appear early, the first being found in the 17 day specimens, beginning as single syncytia and gradually enlarging as time goes on, until miliary tubercles are formed. The simplest of these lie within and not on the omentum, between the peritoneal layers; their syncytia contain ink alone. After 4 weeks they are, of course, more abundant, and the ink still predominates, although a few blue granules are demonstrable in some of the single cells near the periphery which are doubtless wandering macrophages. The taches laiteuses are free from ink and from tubercles. After 5 weeks tubercles can be found penetrating the peritoneal covering, which is broken through and shows dissociated and swollen cells. The general impression obtained is similar to that gained from a study of an ulcer of the intestine; the mesothelial cells are apt to be thickened and piled up at the edges of the lesion, which is composed of larger, paler cells

containing ink and forming a mass that extends well into the connective tissue of the omentum. The mesothelial cells are blue-stained and readily distinguishable from the epithelioid cells as long as they remain well preserved; once they begin to degenerate they are more difficult to distinguish, as they lose the blue granules. It seems probable, from what has just been described, that the tubercles here are formed primarily from the endothelium of the omental capillaries, near which they are first found. The macrophages probably join in the process as it progresses, while the mesothelial tissue appears to be, at the most, sympathetically involved.

Lesions in Lymph Nodes.

Enlarged lymph nodes were regularly found in the axillæ and groins of the rabbits, as well as the peribronchial groups, and were always excised and examined. During the first 3 weeks the medulla is very edematous and its sinuses are filled with large cells of the macrophage type which are deeply stained with Niagara blue and contain phagocytosed cellular debris. They increase in number and size and, in the 17 day specimens, fuse to form free lying syncytia. They do not form tubercles, and carbolfuchsin shows them to be filled with short, plump, reddish rods, totally unlike tubercle bacilli and possibly representing mitochondria. The lymphoid reticulum is stained deeply with Niagara blue, and there is no ink present, except in or near vessels of the cortical nodules.

Tubercles are first found in the 4 week specimens. They are composed of large, pale, anastomosing cells with an evenly distributed amount of carbon granules, which are very fine, but still perfectly evident (Fig. 5—after 5 weeks). Small dilated capillaries are usually demonstrable in their immediate vicinity, five with ink in the endothelium and blood corpuscles in the lumina being found at the periphery of one such tubercle. The tubercle reticulum is suggested in the 4 week specimens, and is well formed and easily demonstrable in the 5 and 7 week preparations. Although the lymphoid reticulum is everywhere deeply stained with Niagara blue, little or none is found in the tubercles. In the 7 week preparations the medulla is nearly free from the large, free cells, and the tubercles show much more ink than those of the earlier stages.

Thus less carbon is deposited in the tubercles formed in the lymphoid tissue of the spleen and lymph nodes than in those of the other organs, with the exception of the kidney. This is probably due to the fact that these tissues have little affinity for carbon and, in the case of the lymphoid tissue, are poorly supplied with blood capillaries. Where the latter abound, plenty of ink is found, as in the lungs, liver, and splenic pulp. It is interesting that, in both spleen and lymph nodes, the large, free lying phagocytes of the pulp or medulla appear to migrate into the lymphoid tissue, for as soon as the administration of the dyes ceases, they leave the sinuses, while the lymphoid tissue becomes full of carbon-bearing cells. That the blue granules are not seen in the lymph nodules in this case is possibly due to the fact that they are more diffusible and can be excreted by the cells that contain them and then be eliminated through the blood and urine, the latter always being of a bluish green color. The carbon seems to be more difficult to remove and is probably retained until the disintegration of the cells containing it sets it free for other phagocytes to take up.

DISCUSSION AND SUMMARY.

A review of the findings in this series of experimental inoculations brings out clearly two points: (1) the specific lesion of the miliary type is composed chiefly of cells of endothelial origin, apparently coming, for the most part, from the walls of the small capillaries near it; and (2) these cells are capable of forming the reticulum of the tubercles and hence collagen. In Hueck's paper, which has already been referred to, the origin and development of the vascular systems are discussed as a subdivision of the mesenchyma. Hueck states that he considers mesenchymal cells of the endothelial type capable of forming collagen fibrils, even in adult life, but indicates that he would limit this attribute more or less strictly to the endothelium of the capillaries. He points out that no "silver lines" can be demonstrated in the capillary endothelium, while they are readily shown in the endothelium of the larger, more specialized vessels; the capillary endothelium is more syncytial, less differentiated, and hence capable of assuming rather varied forms. Hueck's conclusions are in agreement with the findings of the present paper which emphasizes a point that

has been stressed in each of the reports of the series; namely, the importance of the capillary endothelium as distinguished from the endothelium in general.

Rössle and Yoshida several years ago claimed that endothelial cells could produce collagen fibrils by a process of metaplasia. It seems unnecessary, in the light of what has just been said, to apply the term metaplasia to this phenomenon. These cells evidently represent a cytological element in the body that is intended for the process of repair and is unusually plastic, being able to assume varied functions. It is, as it were, a persisting mesenchyma; if this was not so and there were no polyblastic cells present, the process would be slower and less effective. Hueck's ideas on the impregnation of "indifferent fibrillæ" with collagen, elacin, or other substances are also in accord with the staining phenomena observed in the formation of the tubercle reticulum. There is at first a fibrillar or retiform structure to the cytoplasm of the epithelioid cells, without a definite collagen staining reaction. The fibrils can be seen but their color at first does not differ materially from that of the cytoplasm. Gradually, however, they take on a faint pink stain with acid fuchsin or phosphotungstic acid-hematoxylin, or a faint turquoise-blue with aniline blue. These colors increase steadily in intensity as the fibrils become thicker and more evident. The changes from black to brown in the Bielschowsky-Maresch technique are analogous.

That Niagara blue does not appear in the epithelioid cells to any extent must mean one of two things, either the cells do not take it up, or they lose it as soon as they differentiate from the more compact to the larger, paler type. It is certain that they take up and retain the ink through all their phases; it seems doubtful that they have lost many blue granules, as one can seldom demonstrate such a process. If such a reaction did occur the earliest specimens should show groups of cells containing blue granules, while the later stages would show progressive loss of this color. This does not seem to be the case, except in a few instances, when cells with blue granules are occasionally included in tubercles. They are then most often found near the caseous centers.

There may be some question as to the part played by the lymphoid reticular cells in tubercle formation in the lymphoid tissue. If

they do form the epithelioid cells, they must lose their charge of blue dye in so doing and acquire a heavier load of carbon granules. It would seem easier to infer that the epithelioid cells are derived from the endothelium of the scattered vascular capillaries. This would explain the absence of blue granules as well as the smaller amount of carbon present in comparison with that found in the epithelioid cells formed in organs more richly supplied with blood capillaries.

CONCLUSIONS.

1. Miliary tubercles produced in the lung, liver, spleen, kidney, lymph nodes, and omentum of the rabbit by hematogenous tuberculous infection are composed chiefly of cells originating in the capillary vascular endothelium.

2. These cells have a marked affinity for carbon in colloidal suspension, which makes their identification possible.

3. A benzidine dye like Niagara blue, while acting selectively on macrophages, lymphoid reticular cells, renal convoluted tubular epithelium, and free polymorphonuclear leucocytes, is not found in the tubercles to any appreciable extent, unless there is necrosis, when the staining is diffuse.

4. The tubercle reticulum is composed of fibrils resembling connective tissue fibroglia and collagen fibers in every way and produced not only by fibroblasts, but by the endothelial cells themselves, after these have migrated from the vessels, differentiated into epithelioid cells, and formed tubercles. This process is well advanced 5 weeks after inoculation.

BIBLIOGRAPHY.

Downey, H., *Anat. Rec.*, 1916–17, xi, 350.

Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.

Foot, N. C., *J. Med. Research*, 1919, xl, 353; *J. Exp. Med.*, 1920, *a*, xxxii, 513; 1920, *b*, xxxii, 533.

Goldmann, E. E., *Beitr. klin. Chir.*, 1912, lxxviii, 1.

Hueck, W., *Beitr. path. Anat. u. allg. Path.*, 1920, lxvi, 330.

Kiyono, K., Die vitale Karminspeicherung, Jena, 1914.

Kockel, R., *Virchows Arch. path. Anat.*, 1896, cxliii, 574.

Kon, J., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 492.

Kostenitsch, J., and Wolkow, *Arch. méd. exp. et anat. path.*, 1892, iv, 741.
McJunkin, F. A., *Am. J. Anat.*, 1919, xxv, 27.
Miller, J., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 347.
Miller, W. S., *Am. Rev. Tuberculosis*, 1919–20, iii, 1.
Oppenheimer, R., *Virchows Arch. path. Anat.*, 1908, cxciv, suppl., 254.
Rössle, R., and Yoshida, T., *Beitr. path. Anat. u. allg. Path.*, 1909, xlv, 110.
Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.
Soper, W. B., *Am. Rev. Tuberculosis*, 1917–18, i, 385.
Warren, J. H., *J. Med. Research*, 1917, xxxvi, 225.
Watanabe, K., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 367.
Wechsberg, F., *Beitr. path. Anat. u. allg. Path.*, 1901, xxix, 203.

EXPLANATION OF PLATE 12.

Magnification, × about 350. Fig. 1 is from a paraffin section impregnated by the Bielschowsky-Maresch method; the rest are from phosphotungstic acid-hematoxylin preparations.

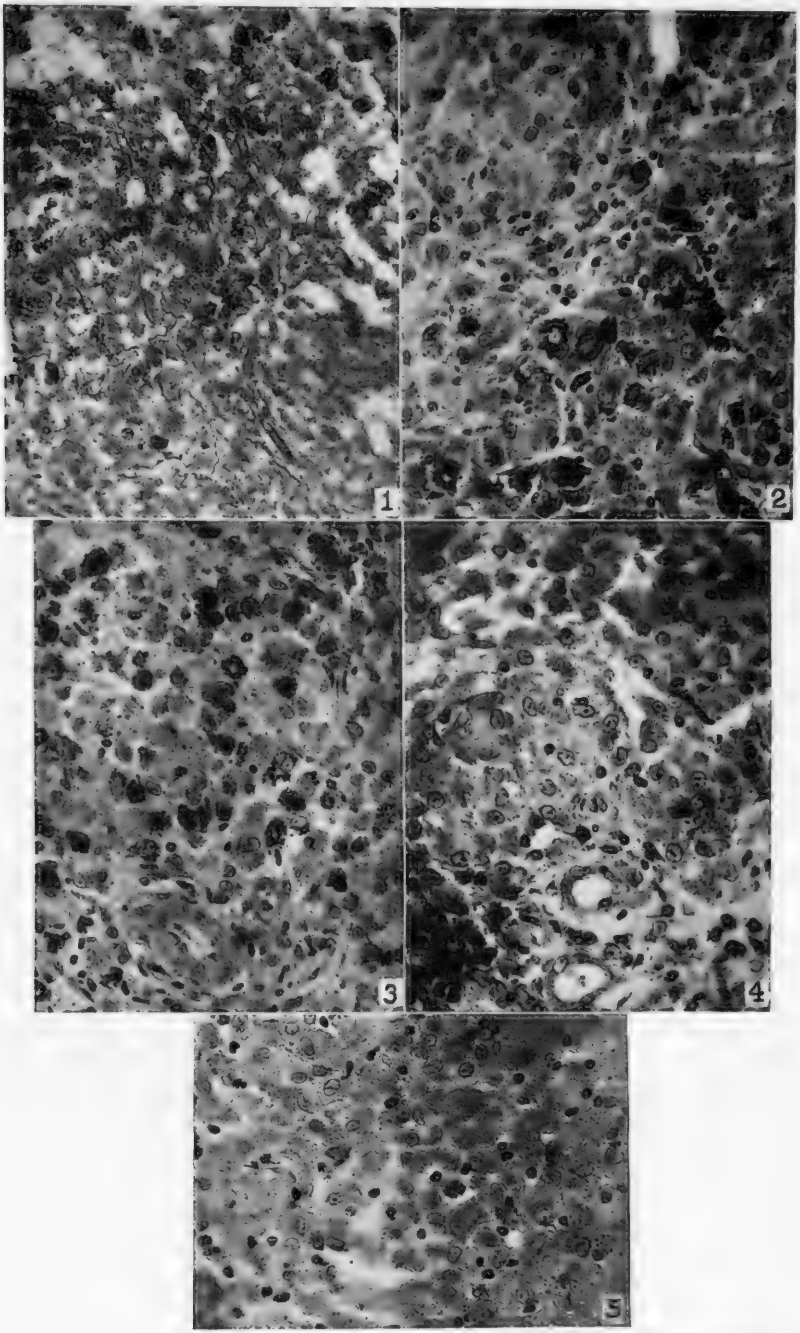
FIG. 1. Reticulum fibrils and carbon particles in the epithelioid cells of a 5 week tubercle in the lung. It will be noted that the fibrils run in the cytoplasm of cells liberally dotted with carbon.

FIG. 2. A 5 week tubercle in the liver, showing the distribution of carbon particles and the peculiar ring-shaped masses referred to in the text.

FIG. 3. A 5 week tubercle in the spleen with more carbon present than is usual in such tubercles. Rings of ink particles are also found.

FIG. 4. A cortical, 5 week tubercle in the kidney. The carbon is rather sparsely distributed, but it can be made out clearly in many of the epithelioid cells.

FIG. 5. Tubercle in the same stage of development in a lymph node. The carbon is very finely divided and still more sparsely distributed, but several cells have a moderately heavy deposit, and careful search reveals several granules in most of the cells.



(Foot: Endothelial reactions. IV.)

CONDITION OF THE CAPILLARIES IN HISTAMINE SHOCK.

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PLATES 13 TO 17

(Received for publication, November 5, 1920.)

The recent work of Dale, Laidlaw, and Richards has brought into prominence the idea that the phenomena observed in shock may have their origin in a primary peripheral circulatory failure. These investigators have used the discovery that histamine (β-iminazolylethylamine) injected intravenously in minute quantities brings about promptly, in certain animals, a condition which is strikingly similar to and apparently identical with the clinical condition designated by the term shock. The blood pressure falls to a very low level and remains there; the pulse becomes small and feeble; the respirations are shallow, irregular, or gasping; the superficial reflexes are active, but a dulling of the sensibility permits the discontinuance of the anesthetic without discomfort to the animal which lies quietly in an apathetic state. Further points of resemblance between histamine shock and clinical shock can be found in detail in the papers by these workers.

The amino-acid histidine enters into the composition of practically every body protein. Since histamine is derived from this amino-acid by decarboxylization, there arises immediately the idea that histamine, or similar substances, may be formed in the body under certain conditions, especially following extensive destruction of tissues by trauma or by widespread inflammatory processes, and that the entry of such substances into the blood stream may be the fundamental cause of the shock which accompanies these conditions. At present, however, in spite of certain suggestive experimental evidence such ideas are entirely speculative, and the present work is not concerned with the question whether histamine and allied substances cause clinical shock. We are interested here rather in a study of the mode of action of histamine which confers upon it its shock-producing potentialities.

The action of histamine has been studied by Dale and his coworkers[1,2] who concluded that the base exerts upon all capillary endothelium a poisonous local effect which results in a dilatation of the lumen of the capillary and an increased permeability of the endothelial wall. The first of these effects is assumed to

[1] Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918–19, lii, 110.

[2] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1918–19, lii, 355.

bring about an accumulation of blood within the capillary system and a consequent inadequate venous return to the heart (". . . the shock-like failure of the circulation is due to accumulation of the blood in relaxed capillaries . . . the flow through the capillaries becomes ever slower and the tendency to stagnation greater, till ultimately only a relatively small part of the available blood is effectively circulating."[2] The increased permeability of the capillary walls is assumed to favor the escape of plasma from the vessels and so to impose an actual oligemia upon the already enfeebled circulation. While Dale and Laidlaw[2] are "convinced that general dilatation of the capillaries and loss of plasma from the blood are main factors in the production of the shock," they were unable to observe directly the reactions of capillaries subjected to the influence of histamine, but deduced their conclusions from other considerations, stating: "Several attempts were made to observe changes in the calibre of capillaries under a moderate power of the microscope, without any result to which we could attribute importance." Dale and Richards[1] had written previously: "We made several attempts to measure changes in the calibre of blood vessels—arterioles and capillaries—in the mesentery by direct observation with the microscope, histamine solution being directly applied to the area under observation. The results were not sufficiently convincing to ourselves to make it desirable to record them. Later experience of the ease with which this vasodilator action is impaired, by temporary interference with the circulation through an organ or exposure to cold, made it clear that satisfactory observations of this kind would need a specially elaborated technique."

Since this important theory of shock has as its basis the assumption of accumulation of the blood within a capillary system dilated by the action of an endothelial poison, the present work was begun, at the suggestion of Dr. W. G. MacCallum, in an attempt to study directly the effect of histamine upon the capillary system in order to determine (1) whether histamine is actually an endothelial poison acting locally upon capillaries to produce a dilatation of their lumina, (2) whether the capillary bed during histamine shock undergoes a general dilatation of a degree sufficient to endanger the circulation, and (3) whether such a dilatation precedes or follows the circulatory failure occurring in shock.

EXPERIMENTAL.

The omentum was chosen as the site most suitable for observation because its transparency favors microscopic study in fresh and fixed preparations and especially because the local action of histamine was

to be studied. Since the capillaries of the omentum are for the most part practically surface capillaries, they can easily be brought into contact with locally applied histamine solutions.

Effect of Histamine Studied Microscopically in Vivo.

An attempt was first made to study microscopically, *in vivo*, the effect of histamine upon the capillary circulation of the omentum.[3] Any satisfactory technique for such a study must safeguard the capillary bed under observation from at least four sources of error—trauma, mechanical interference with the circulation, temperature changes, and evaporation of moisture. In order to protect the field from abnormal temperature changes a box large enough to accommodate an anesthetized cat was constructed of wood, lined with asbestos, and equipped with a thermostat. The chamber was fitted with a glass window, a movable opening for the barrel of a microscope, and felt arm sleeves through which work could be carried on within the box while it was entirely closed to maintain a constant temperature. A microscope, from which the base and substage had been removed, was attached to a horizontal bar clamped to an upright rod so that it could be raised or lowered, advanced or retracted to the desired position. The stage was covered with a thick glass plate, and a minute electric globe taken from a small pocket flash-light was fitted beneath the stage and served for illumination; the amount of heat given off by this tiny lamp was too slight to be measured by an ordinary thermometer placed on the glass plate above it.

Dale and his coworkers found cats especially susceptible to the action of histamine. This was the animal used, therefore, in all the present experiments. A cat was anesthetized with ether, tracheotomized, and connection established with an ether respiration bottle by means of a tracheal cannula. The animal was then placed within the box in which the atmospheric temperature was maintained at about 37°C. The abdomen was opened along a midline incision, the edges of which were held apart by small Balfour retractors. The intestines were covered with pads soaked in normal saline solution; the glass stage was warmed to 37° C. by immersion in normal saline

[3] All operations were performed under ether anesthesia.

solution kept at that temperature. The microscope was now advanced and lowered so that the stage occupied a position within the abdominal opening on a level with the intestines. The omentum was then gently lifted onto the stage of the microscope, care being taken to avoid unnecessary stretching, twisting, pulling, or any manipulation which might traumatize the omentum or disturb its circulation even momentarily. The stage of the microscope was made to occupy such a position that the omentum could be lifted upon it with a minimal displacement from the normal situation. During the period of observation the omentum was continually kept moist by means of capillary pipettes from which normal saline solution flowed, warmed to body temperature. Thus the omentum lay upon the glass stage of the microscope scarcely disturbed from its normal position, was protected against the effects of drying, and its normal temperature was maintained. Throughout all the observations the omentum was handled as little as possible and always with extreme care.

With the low power objective the blood can be watched coursing through the capillaries and the smaller arterioles and venules of the omentum. Capillaries may easily be seen which have diameters so small that they permit the passage of only one red corpuscle at a time. The omentum may be shifted gently upon the stage until a capillary bed which is suitable for study comes into the field. A field which contained a small arteriole and venule, as well as a rich anastomosis of capillaries of different diameters, was usually chosen. Any individual vessel selected for observation was measured at intervals with an eyepiece micrometer in order that any variations in diameter might be detected. While the omentum lay undisturbed upon the stage of the microscope, capillaries were observed as long as 30 minutes, during which time there occurred no measurable changes in their diameters.

Local Application of Histamine.—After a field had been chosen for observation, the vessels were watched for several minutes in order to acquire an impression of the rate of flow through them, and the diameters of several selected capillaries were measured. Then, while the observer kept a careful watch over these measured capillaries and the blood stream flowing through them, an assistant, using a capillary pipette, flooded the field with a solution composed of his-

tamine[4] dissolved in normal saline solution warmed to body temperature. Solutions of histamine ranging in strength from 1:500,000 to 1:500 were in this manner applied locally to the capillaries of the omentum in a large number of experiments. Careful inspection and measurement disclosed no change whatever in the diameters of the capillaries or in the rate of flow through them, nor were any capillaries opened up which were not visible before the application of histamine. Under the conditions of these experiments histamine locally applied produced no detectable change in the appearance of the capillary circulation.

Histamine Shock.—In another series of experiments, while a field of capillaries was kept under observation, the animal was thrown into shock by an intravenous injection of 4 mg. of histamine, blood pressure tracings being taken from the carotid artery. As the blood pressure fell the rate of flow through the capillaries became slower, until the stream was moving in a strikingly sluggish manner; but again, as with local applications of histamine, no widening of the capillaries could be detected.

These experiments demonstrated that a pronounced slowing of the capillary stream accompanies the fall in blood pressure which histamine produces when injected intravenously. Two possibilities were suggested in explanation of the fact that the local application of histamine did not affect the size of the capillaries in these experiments. Either histamine exerts no effect upon the diameters of capillaries, or else the handling of the omentum necessary to bring it under the microscope, in spite of the care with which this was done, inflicted sufficient trauma to bring about either a capillary paralysis and dilatation or a loss of capillary irritability, with the result that the vessels could not be dilated further by the action of histamine or any other agent. Experiments were, therefore, carried out in which

[4] The histamine preparation used was the dihydrochloride of the base supplied by the Hoffmann La Roche Chemical Works of New York. The activity of the stock supply was tested from time to time upon the virgin uterus of the guinea pig and was found to produce the characteristic contractions at each test. The potency of this preparation was also exhibited in the numerous animal experiments performed during this study, in which it was used to induce shock. Fresh solutions were always prepared before every experiment.

irritants and alleged capillary dilators were applied locally to omenta under microscopic observation.

Local Application of Inflammatory Agents.—Among the substances used were cantharides, turpentine, croton oil, gold and sodium chloride, amyl nitrite, and chloroform. It is well known that irritants locally applied to the web of the frog's foot bring about a marked dilatation of the capillaries,[5] and that in mammals the intraperitoneal injection of these substances produces a dilatation of the peritoneal capillaries during the resulting inflammatory reaction. It was assumed, therefore, that, if the normal reactivity of the capillaries under observation in these experiments was undisturbed, the local application of such substances would be followed by definite changes in the capillary diameters. Some of the irritants brought about a stasis of blood in the capillary area to which they were applied—apparently a result of osmotic disturbances. In no instance was there seen any definite dilatation of the capillaries which, in some experiments, were observed as long as 30 minutes following the application.

Effect of Histamine Studied in Fixed and Stained Preparations.

Since the local application of such inflammatory agents failed to produce a detectable capillary dilatation, it seemed that the conditions of the experiment must have brought about some alteration of capillary irritability, and an attempt was made to determine what effect the handling of the omentum in these observations exerts upon the capillaries. In a series of experiments a midline incision was made in the abdomen of normal cats under anesthesia, and the abdominal cavity was immediately flooded with a fixative. The fixative used in all experiments was a modification of Zenker's fluid, formaldehyde being substituted for glacial acetic acid. The omentum was then quickly cut along the line of attachment and transferred to a dish of the fixative where it was left for 24 hours. It was washed, spread over large glass slides, and stained with hematoxylin

[5] The web of the frog's foot, while offering ordinarily a standard and convenient field for capillary observation, was unsuited for study in these experiments, because the frog is extremely resistant to the shock-producing effects of histamine.

and Van Gieson's stain. In such a preparation the arterioles, venules, and capillaries are distinguished in minute detail. Since the omentum was, in this manner, fixed before being subjected to any manipulation or exposure, these slides were assumed to reveal the normal appearance of the omental capillaries. In another series of experiments, after the abdomen had been opened, the omentum was lifted to the stage of the microscope with all the care and precautions described above. It was at once fixed by flooding with the fixative, and was then treated in the same manner as the normal control specimens.

Microscopic comparison of the capillaries in the omenta of these two series at once revealed striking differences in appearance. The capillaries and the smaller arterioles and venules of the manipulated omenta were distinctly dilated, tortuous, and engorged with blood; furthermore, there was an actual increase in the number of visible capillaries in the handled omenta. It is clear from a study of these slides that many of the fat cells within the fat streaks are bounded by capillaries, that at any given time, under normal conditions, only a relatively small number of these capillaries are open channels for the blood stream, many lying collapsed and closed, and that handling the omentum opens up the collapsed capillaries either by causing a loss of tone through paralysis or by stimulating them to dilate.

The opening up of occult capillaries is seen with especial distinctness along the arterioles and venules which run through the connective tissue between the fat streaks. Clumps of mononuclear cells lie along the course of many of these vessels and form cellular sheaths about them. In the normal omenta these cellular areas are apparently avascular. It is only occasionally that a delicate capillary loop can be seen outlined between the cells. But in the handled omenta these cell clumps are invariably traversed by rich, engorged capillary plexuses, so that the arteriole and venule are bordered by a network of capillaries which can easily be traced to their origins from the arteriole and venule. This appearance is so striking that the handled omenta can be quickly distinguished from the normal omenta by an inspection of these areas.

These observations are entirely in accord with Krogh's[6] demonstration that normally, at any given time, only a relatively small

[6] Krogh, A., *J. Physiol.*, 1918–19, lii, 457.

portion of the entire capillary bed is an open channel for the blood stream, many capillaries remaining collapsed and microscopically invisible; and that these collapsed capillaries open up under conditions which excite their dilatation.

It was evident from a study of these slides that the capillaries were dilated by the manipulation necessary for the placing of the omentum upon the microscope stage; and this fact made it clear that no trustworthy conclusions concerning the effect of histamine upon capillaries could be drawn from such microscopic observations *in vivo*.

Local Application of Histamine.—Since the method of fixation of the omentum had yielded such distinctive results, it was utilized in an attempt to study the reaction of the capillaries under the influence of histamine. Two series of experiments were carried out. The animals in the first series were treated as follows: After 20 minutes anesthesia the abdomen was opened along the midline and 10 cc. of normal saline solution warmed to 37°C. were immediately poured over the omentum. The edges of the incision were quickly drawn together and held closed. The omentum was not handled in any way. $1\frac{1}{2}$ minutes after the saline solution had been applied, the edges of the incision were retracted and the omentum was fixed by flooding with the fixing solution. It was quickly removed and permanent stained preparations were made as in the previous experiments.

Microscopic study of these omenta showed that the capillaries, arterioles, and venules presented an appearance in no way distinguishable from those of the previous experiments in which the omenta were immediately fixed upon opening the abdomens. It was apparent, then, that the application of saline solution had not affected the capillaries in any way, and the blood vessels in omenta of this series were considered to exhibit the normal vascular appearance (Figs. 1 and 2).

In the second series of experiments the technique was the same as in the first, except in one respect. The period of anesthetization was the same, the solutions, the temperatures, and the length of time between the saline applications and fixation were identical, but in these experiments histamine was dissolved in the saline solutions, so that the histamine proportion in various experiments ranged from 1:1,000 to 1:20,000. The omenta from these two series of experi-

ments thus afforded for study capillaries which had been subjected to local applications of histamine, and controls in which, under the same conditions, normal saline solution had been applied locally instead of histamine. Microscopic study of these preparations showed that the capillaries of the omenta which had been brought in contact with histamine were very markedly dilated, tortuous, and engorged with blood (Fig. 3) in comparison with the capillaries in the normal controls. There was also seen a distinct increase in the number of visible capillaries. The dilatation was not confined to the capillaries but included the smallest arterioles and venules at the periphery of the capillary bed. The local application of histamine clearly brings about a marked dilatation of both the visible and occult capillaries. That this effect is local was demonstrated by blood pressure tracings taken during the experiments. The pressure was maintained, with the usual slight fluctuations, at a constant normal level, indicating that during the period of contact of the capillaries with histamine not enough of the base had been absorbed to exert the slightest depressor action upon the circulation. When strong histamine solutions were used in the local applications a fall of blood pressure occurred, indicating sufficient absorption to produce the characteristic depressor effect, but the series examined for the purely local effects of the base comprised the omenta only from animals which had exhibited no blood pressure disturbance during the application.

Histamine Shock.—In another series of experiments the animals were thrown into shock by intravenous injections of histamine dissolved in 2 cc. of normal saline solution, 2 mg. of the base being used per kilo of body weight. When the blood pressure had fallen to the shock level the abdomen was opened and the omentum immediately fixed as described above. As controls, the omenta of animals which had been given an intravenous injection of 2 cc. of normal saline solution instead of histamine were fixed, the time between the injection and the fixation of the omentum being the same in the two series. The omenta of these controls exhibited a vascular picture differing in no way from that of the normal controls previously described. The capillaries and smaller arterioles and venules of the shocked animals, on the other hand, were definitely dilated and

engorged with blood; and here again it was clear that many occult capillaries had been opened up (Figs. 4 and 5). A study of these slides made it evident that a widespread peripheral vascular dilatation of such a degree could easily accommodate an amount of blood large enough seriously to impair the circulation.

These experiments demonstrated that histamine exerts a local dilating effect upon capillaries and that during histamine shock the blood stream moves sluggishly through a dilated, engorged capillary bed. It was now of importance to determine the relation of the capillary dilatation to the fall in blood pressure which occurs during shock.

Relation of Capillary Dilatation to Blood Pressure Fall in Shock.—Experiments were made in which the abdomens were opened and the omenta fixed at various intervals following the injection of histamine. It was found from a study of these preparations that the capillary dilatation had begun in the omentum taken 15 seconds after the injection, before the blood pressure had fallen to the shock level. The progression of the dilatation to a maximum could be followed by a study of the omenta taken at intervals of 15 seconds. No recovery from this peripheral vascular dilatation has been encountered; omenta fixed as late as an hour after the onset of shock have shown the persistence of the dilatation, which apparently must be looked upon as a paralytic loss of capillary tone. The dilatation was uninfluenced by the brief secondary rise in blood pressure which characteristically follows the injection of histamine, and which Dale and Laidlaw have attributed to a constriction of the pulmonary arteries. Omenta fixed at the summit of this rise exhibit the vascular dilatation. This fact makes it clear that the capillary dilatation and engorgement occurring during shock are not a mere result of low blood pressure; the fall of blood pressure is more properly to be attributed to the peripheral vascular relaxation.

There has been ample opportunity during this study to confirm the observations of Dale and Laidlaw[2] regarding the excellent functional condition of the heart in histamine shock. The fall in blood pressure cannot be attributed to cardiac failure. It has frequently been observed, in the present experiments, that during unrecoverable shock the heart efficiently discharges relatively large amounts of

saline solution injected into the jugular vein. In a number of instances repeated intravenous injections of adrenalin have been administered to animals in deep shock, and the heart has always worked with remarkable efficiency during the height of the blood pressure rise. Death during shock has always resulted from respiratory rather than cardiac failure.

The inadequate venous return to the heart has also been observed, and an inspection of the great veins makes it clear that the blood is not accumulating in that portion of the vascular system. The present experiments demonstrate that histamine exerts a local dilator effect upon capillaries and that during histamine shock there are marked widening and engorgement of the capillary bed. The capillary dilatation during shock is accompanied by a fall in blood pressure, but persists independently of subsequent blood pressure variations. These facts are entirely in accord with the view of Dale and Laidlaw that the fall of blood pressure during histamine shock is due to a general loss of capillary tone produced by the poisonous local action of the base.

CONCLUSIONS.

1. Histamine exerts a local dilator effect upon capillaries and upon the smallest arterioles and venules which border the capillary system. There occurs also an opening up of large numbers of capillaries of which no trace can be seen before the application of histamine.

2. When injected intravenously in amounts sufficient to produce shock, histamine causes a quickly progressive dilatation of both the visible and the occult capillaries and of their immediately adjacent arterioles and venules, all of which become engorged with blood that moves through them in a strikingly sluggish manner. The circulatory failure which characterizes histamine shock results from the dilatation of the peripheral vascular bed.

EXPLANATION OF PLATES.

The five photomicrographs were taken with the same magnification.

PLATE 13.

FIG. 1. Fat streak in the omentum. Local application of normal saline solution.

PLATE 14.

FIG. 2. Arteriole and venule in the normal omentum showing the apparently avascular cell sheath.

PLATE 15.

FIG. 3. Fat streak in the omentum. Local application of histamine. Blood pressure normal.

PLATE 16.

FIG. 4. Fat streak in the omentum during histamine shock.

PLATE 17.

FIG. 5. Arteriole and venule in the omentum during histamine shock, showing capillary network throughout the cell sheath.

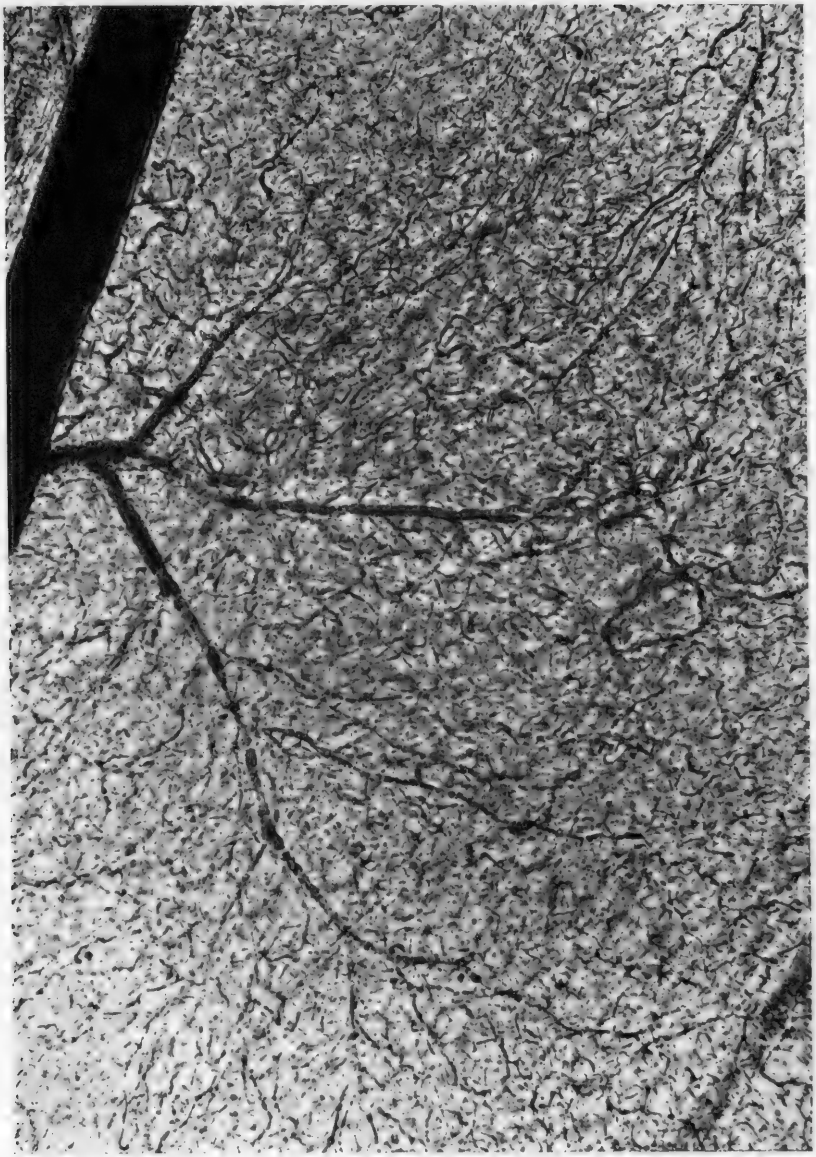

FIG. 1.

(Rich: Capillaries in histamine shock.)

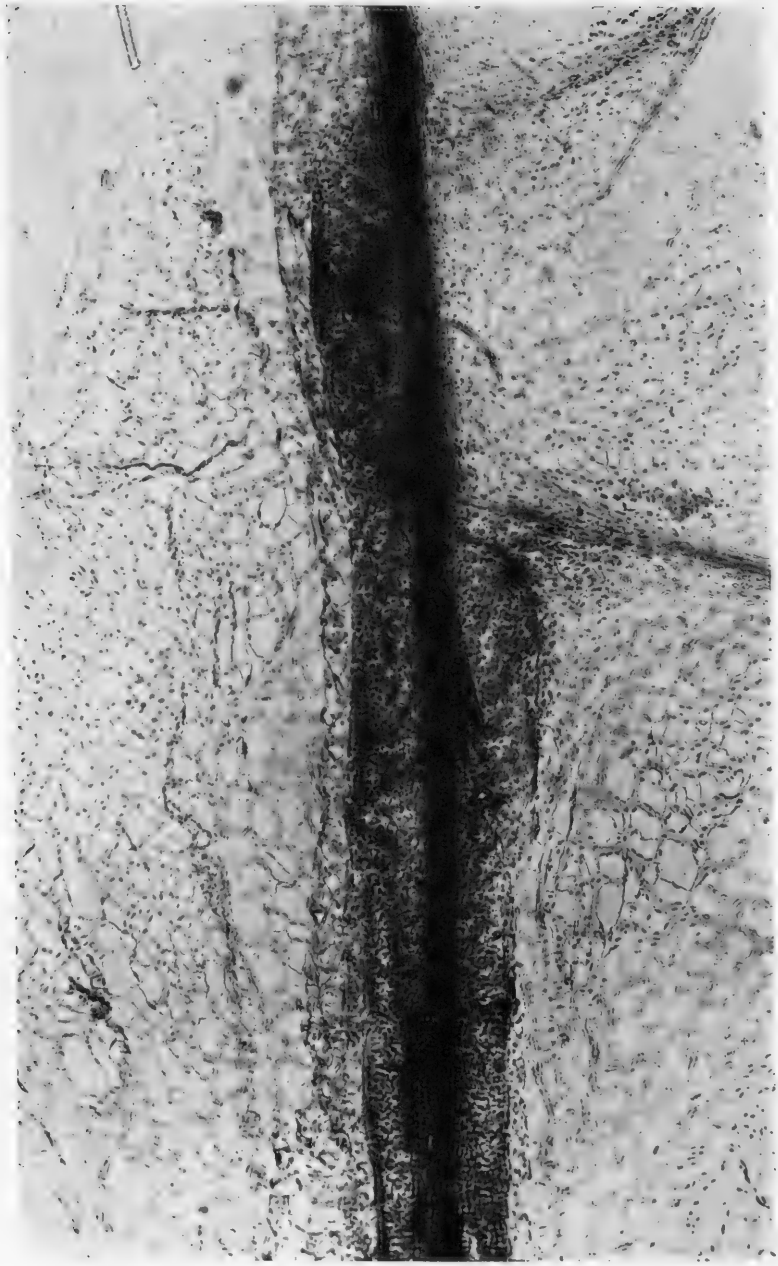

FIG. 2.

(Rich: Capillaries in histamine shock.)

FIG. 3.

(Rich: Capillaries in histamine shock.)

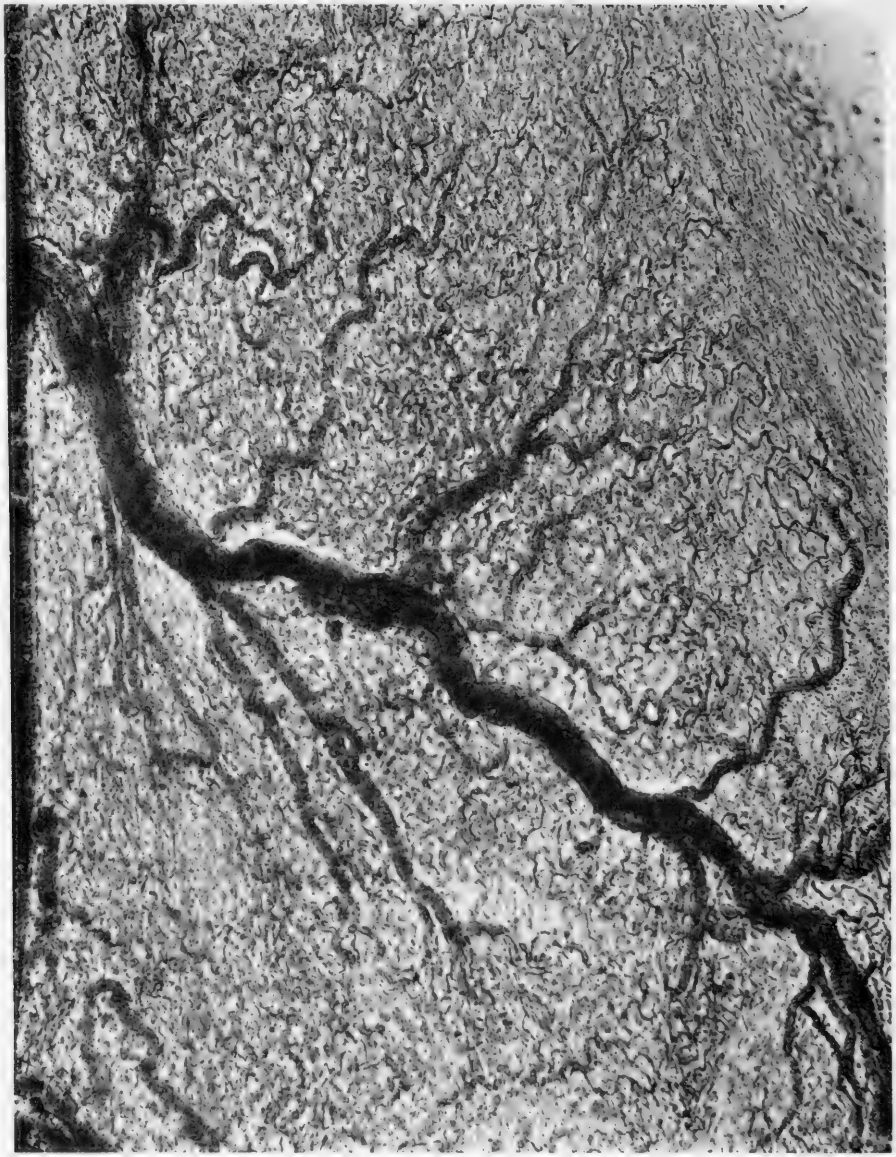

FIG. 4.

(Rich: Capillaries in histamine shock.)

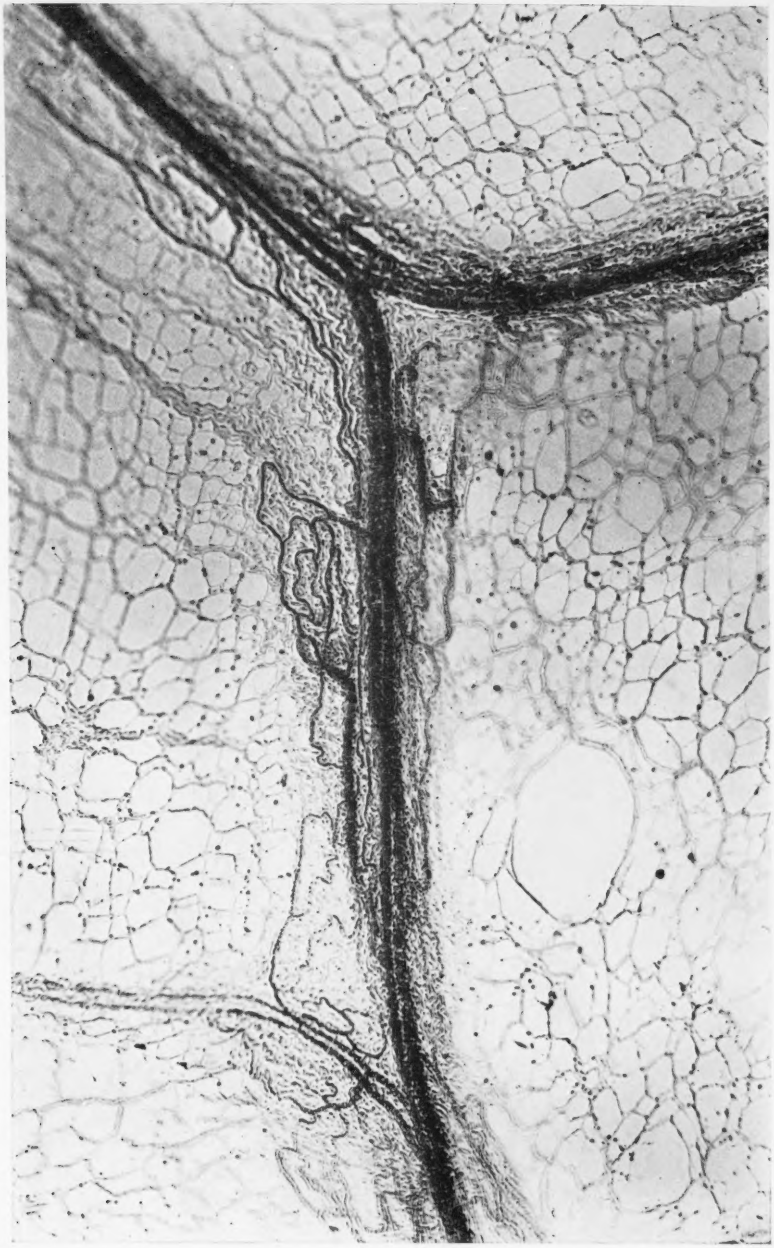

FIG. 5.

(Rich: Capillaries in histamine shock.)